Tampere University

LAURA ALIISA SAARIMÄKI

Toxicogenomics Data for Chemical Safety Assessment

From Intrinsic Characteristics to Functional Potential

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ACADEMIC DISSERTATION
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Tampere, February 2024

Laura

ABSTRACT

The modern world surrounds us with a vast sea of chemicals of which a large majority remains uncharted in terms of their potential hazard for human health and the environment. At the same time, the rapid introduction of new chemicals necessitates a delicate balance between innovation and safety, presenting one of the key challenges of the 21st century.

Chemical safety assessment has been long focused on resource-intensive and ethically challenged animal experiments. Furthermore, the traditional approach focuses on phenotypic endpoints, providing limited insight into toxicity mechanisms which impedes the development of chemicals that are safe and sustainable by design (SSbD). Despite major efforts to advance *in vitro* and *in silico* alternatives for chemical safety assessment, the first generation of these non-animal approaches present similar challenges as their *in vivo* counterparts. This has resulted in major initiatives to shift the focus towards mechanistic toxicology, enabling a deeper understanding of chemical hazards. This mechanistic approach is fuelled by the introduction of adverse outcome pathways (AOPs) and toxicogenomics, offering unprecedented insights into the molecular underpinnings of chemical-induced toxicity. While the value of this mechanistic venture is broadly recognised, toxicogenomics-based evidence is not systematically integrated into chemical safety assessment.

Hence, the foundational premise of this dissertation lies in the recognition of the multifaceted challenges that surround the utilisation of toxicogenomics data in chemical safety assessment. These challenges were characterised through three critical aspects of toxicogenomics data: its intrinsic characteristics, functional properties, and translational potential. The intrinsic characteristics, defined as the FAIRness (Findability, Accessibility, Interoperability and Reusability) and quality of data, were investigated, and addressed through systematic data curation and annotation. This enabled a comprehensive review of the current state of toxicogenomics data, resulting in a resource with improved FAIRness and a robust foundation for subsequent analytical endeavours. Similarly, this effort established a systematic link between toxicogenomics-based evidence and the AOP framework, empowering the functional properties of both data types.

Innovative methodologies and approaches to data analysis form the cornerstone of the functional properties of data, aiming at the extraction of meaningful insights from complex, high-dimensional toxicogenomics datasets. By harnessing advanced computational techniques and the link established between AOPs and toxicogenomics, this dissertation further sought to distinguish subtle molecular signatures and discern the intricate interplay between chemicals and biological systems. This was exemplified by a model of a dynamic dose-dependent mechanism of action that revealed crucial mechanisms related known long-term adverse effects of multi-walled carbon nanotube exposure in a short-term *in vitro* exposure.

Finally, a pivotal facet of this research lies in the translation of toxicogenomics-derived evidence into biologically meaningful events that are comprehensible to a broader audience. Bridging the gap between raw data and actionable insights, this dissertation endeavored to provide a tangible link between molecular alterations and their potential implications for human health through the translation of toxicogenomics-based evidence into mechanistic new approach methodologies (NAMs). This dissertation highlighted how the intrinsic characteristics and functional properties of toxicogenomics data enable its translational potential, resulting in the AOP fingerprint and *in vitro* biomarkers for the evaluation of profibrotic potential of chemicals.

Ultimately, the results of this research have the potential to propel the field of chemical safety assessment forward by elucidating the intrinsic characteristics, functional properties, and translational potential of toxicogenomics data. By synergistically employing data curation, advanced analytical methodologies, and translational approaches, this dissertation endeavours to enhance the applicability of toxicogenomics in the broader context of chemical safety evaluation, thus contributing to the safeguarding of public health and the environment.

TIIVISTELMÄ

Nykyaikainen maailma ympäröi meitä valtavalla määrällä kemikaaleja, josta suurin osa on edelleen arvioimatta niiden mahdollisten terveys- ja ympäristövaikutusten osalta. Samalla uusien kemikaalien nopea kehittäminen ja käyttöönotto vaatii taidokasta tasapainottelua innovaation ja turvallisuuden välillä, muodostaen yhden nykypäivän keskeisistä haasteista. Kemikaalien turvallisuusarviointi on pitkään keskittynyt kalliisiin ja työläisiin eläinkokeisiin, joiden eettisyys ja relevanssi usein kyseenalaistetaan. Lisäksi perinteinen lähestymistapa kemikaalien turvallisuusarviointiin keskittyy havaittaviin fenotyyppimuutoksiin, tarjoten rajallisen käsityksen lopputulokseen johtavista mekanismeista ja molekyylitason vasteista. Tämä puolestaan rajoittaa kehitystä kohti uusia kemikaaleja, jotka suunnitellaan turvallisiksi ja kestäviksi alusta alkaen (safe and sustainable by design, SSbD). Vaikka merkittäviä ponnisteluja on tehty niin *in vitro* - kuin *in silico* -vaihtoehtojen edistämiseksi kemikaalien turvallisuusarvioinnissa, näiden eläinkokeita korvaavien menetelmien ensimmäinen sukupolvi kohtaa samankaltaisia haasteita kuin niiden eläinperusteiset vastineet. Tämä on johtanut merkittäviin globaaleihin aloitteisiin siirtää painopiste kohti mekanistista toksikologiaa, joka mahdollistaa syvällisemmän ymmärryksen kemikaalien mahdollisista haitoista. Mekanistinen toksikologia perustuu perusteelliseen ymmärrykseen kemiallisten altistusten aiheuttamista mekanismeista ja vasteista. Molekyylitasolla näitä mekanismeja tutkitaan toksikogenomiikan keinoin ja laaja-alaisemmin mekanismit voidaan kuvata käyttäen apuna haittavaikutusreittejä (adverse outcome pathways, AOPs). Vaikka tämän mekanistisen lähestymistavan potentiaali tunnustetaan laajasti, toksigenomiikkaan perustuvaa näyttöä ei vielä käytetä systemaattisesti osana kemikaalien turvallisuusarviointia.

Tämän väitöskirjan lähtökohta piileekin niissä monitahoisissa haasteissa, jotka vaikuttavat toksigenomiikan käyttöön kemiakaalien turvallisuusarvioinnissa. Tässä väitöskirjassa näitä haasteita tutkittiin toksikogenomiikkaan ja sen dataan liittyvillä kolmella kriittisellä osa-alueella: sen sisäiset ominaisuudet, toiminnalliset ominaispiirteet sekä sen translationaalinen potentiaali. Datan sisäisiin ominaisuuksiin

määriteltiin kuuluvaksi sen FAIR-luonteisuus (lyhenne sanoista Findability, Accessibility, Interoperability ja Reusability), käytettävyys ja laatu. Näitä ominaisuuksia tutkittiin ja käsiteltiin systemaattisella datan kuratoinnilla ja merkinnällä, mikä mahdollisti myös datan nykytilanteen kattavan tarkastelun. Tuloksena syntyi kokoelma, jolla on parannellut FAIR-ominaisuudet, luoden vankan pohjan seuraaville analyyttisille pyrkimyksille. Lisäksi tämä pyrkimys loi systemaattisen yhteyden toksigenomiikan ja olemassa olevien haittavaikutusreittien välille, vahvistaen molempien datatyyppien toiminnallisia ominaisuuksia.

Innovatiiviset menetelmät ja lähestymistavat datan analysointiin muodostavat toiminnallisten ominaisuuksien perustan. Näiden tavoitteena on löytää merkityksellisiä oivalluksia monimutkaisesta ja moniulotteisesta datasta, jota tuotetaan toksikogenomiikan keinoin. Tässä väitöskirjassa näitä toiminnallisia ominaisuuksia tutkittiin hyödyntämällä edistyneitä laskennallisia menetelmiä sekä haittavaikutusreittien ja toksikogenomisen datan välille luotua yhteyttä pyrkimyksenä luonnehtimaan ja mallintamaan kemikaalien ja biologisten järjestelmien monimutkaisia vuorovaikutuksia. Esimerkkinä tästä syntyi dynaamisen vaikutusmekanismin malli, jonka avulla voitiin luonnehtia lyhyen *in vitro* -altistuksen avulla niitä mekanismeja, jotka johtavat keuhkofibroosiin pitkällä aikavälillä moniseinäisille hiilinanoputkille altistuttaessa.

Viimeinen osa-alue keskittyi toksigenomiikasta johdetun näytön kääntämiseen biologisesti merkityksellisiksi tapahtumiksi, jotka ovat ymmärrettäviä laajemmalle yleisölle. Toksikogenomiikan datan translationaalinen potentiaali piilee keinoissa kuroa umpeen kuilu raakadatan ja käytännön oivallusten välillä. Tässä väitöskirjassa pyrittiin luomaan tämä konkreettinen linkki molekulaaristen vasteiden ja niiden mahdollisten terveysvaikutusten välille kääntämällä toksikogenomiikan keinoin saatu näyttö mekanistisiksi uuden sukupolven menetelmiksi (new approach methodologies, NAMs). Tuloksissa korostui datan sisäisten ja toiminnallisten ominaisuuksien merkitys translationaaliselle potentiaalille.

Kokonaisuutena tämä väitöskirjatutkimus pyrkii edistämään kemikaalien turvallisuusarvioinnin alaa tutkimalla toksigenomisen datan sisäisiä ominaisuuksia, toiminnallisia ominaispiirteitä sekä translationaalista potentiaalia. Hyödyntämällä datan kuratointia, edistyneitä analyyttisiä menetelmiä ja uusia lähestymistapoja, tämä väitöskirja pyrkii parantamaan toksigenomiikan soveltamista kemikaalien turvallisuusarviointiin laaja-alaisesti, edistäen matkaa kohti terveellisempää ja turvallisempaa tulevaisuutta.

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ABBREVIATIONS

3R	Replacement, reduction, and refinement
AIC	Akaike Information Criterion
AO	Adverse outcome
AOP	Adverse outcome pathway
BMD	Benchmark dose
BMDL	Lower bound of the benchmark dose
BMDU	Upper bound of the benchmark dose
BMR	Benchmark response
cDNA	Complementary DNA
CTD	Comparative toxicogenomics database
DE	Differential expression
DEG	Differentially expressed gene
dMOA	Dynamic dose-dependent mechanism of action
GEO	Gene expression omnibus
GLP	Good Laboratory Practice
ECHA	European Chemicals Agency
ENA	European Nucleotide Archive
ENM	Engineered nanomaterial
EPA	U.S. Environmental Protection Agency
FAIR	Findability, Accessibility, Interoperability and Reusability
FC	Fold change
FDR	False discovery rate
IATA	Integrated approaches for testing and assessment
IDF	Inverse document frequency
KE	Key event
LOAEL	Lowest observed adverse effect level
logFC	Fold change expressed in the log2 scale
MIAME	Minimum information about a microarray experiment
MIE	Molecular initiating event
MINSEQE	Minimum information about a sequencing experiment

MOA	Mechanism of Action
MWCNT	Multi-walled carbon nanotube
NAM	New Approach Methodology
NCBI	National Center for Biotechnology Information
NGS	Next generation sequencing
NLP	Natural language processing
NOAEL	No observed adverse effect level
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
PMI	Phorbol 12-myristate 13-acetate
POD	Point of departure
PPI	Protein-protein interaction
qAOP	Quantitative adverse outcome pathway
QSAR	Quantitative structure activity relationship
RAHC	Reasonably anticipated human carcinogen
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RQN	RNA quality number
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SSbD	Safe and sustainable by design
TEM	Transmission electron microscope
TF	Transcription factor

ORIGINAL PUBLICATIONS

This dissertation is based on the following publications:

I **Saarimäki L. A.**, Federico A., Lynch I., Papadiamantis A. G., Tsoumanis A., Melagraki G., Afantitis A., Serra A. & Greco D. Manually curated transcriptomics data collection for toxicogenomic assessment of engineered nanomaterials. *Sci. Data* 8, 49 (2021).

II **Saarimäki L. A.**, Melagraki G., Afantitis A., Lynch I. & Greco D. Prospects and challenges for FAIR toxicogenomics data. *Nat. Nanotechnol.* **17**, 17–18 (2022).

III **Saarimäki L. A.***, Kinaret P. A. S.*, Scala G., del Giudice G., Federico A., Serra A. & Greco D. Toxicogenomics analysis of dynamic dose-response in macrophages highlights molecular alterations relevant for multi-walled carbon nanotube-induced lung fibrosis. *NanoImpact* 100274 (2020).

IV **Saarimäki, L. A.**, Fratello, M., Pavel, A., Korpilähde, S., Leppänen, J., Serra, A. & Greco, D. A curated gene and biological system annotation of adverse outcome pathways related to human health. *Sci. Data* 10, 409 (2023).

V **Saarimäki, L. A.**, Morikka, J., Pavel, A., Korpilähde, S., del Giudice, G., Federico, A., Fratello, M., Serra, A. & Greco, D. Toxicogenomics Data for Chemical Safety Assessment and Development of New Approach Methodologies: An Adverse Outcome Pathway-Based Approach. *Adv Sci (Weinh)* 10, e2203984 (2023).

*Equal contributions

The corresponding Roman numerals are used to refer to each study in the text.

AUTHOR'S CONTRIBUTION

I L.A. Saarimäki collected, preprocessed, and analysed the microarray data, produced the figures, and wrote the manuscript.

II L.A. Saarimäki performed the investigation and wrote the manuscript.

III L.A. Saarimäki performed the RNA quality assessment and the gene expression microarray experiment, as well as preprocessed and analysed the gene expression and the DNA methylation data. L.A. Saarimäki conducted the investigation and data interpretation, produced the figures, and wrote the manuscript.

IV L.A. Saarimäki participated in the design of the study and manually curated the data together with two students whose curation tasks she supervised. L.A. Saarimäki conducted the analysis and investigation, produced the figures, and wrote the manuscript.

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1 INTRODUCTION

Chemicals pervade all aspects of human lives. We are exposed to thousands of chemicals through food, everyday products, medicines, and the environment throughout a lifetime. However, only a fraction of these chemicals has been thoroughly characterised for their potential hazards on human health and the environment (European Environment Agency, 2019). At the same time, novel substances are constantly needed to overcome modern challenges of humankind, which makes balancing sustainable innovation with safety one of the key challenges of the 21st century.

Traditionally, chemical safety has been assessed through animal experiments that focus on exposure properties and observable, phenotypic changes induced by chemical exposures. These assays, however, are time-consuming, costly, unethical, and often not easily translated to human health (Landi et al., 2021; Price et al., 2022; Van Norman, 2019). Similarly, the evaluation of exposure-induced apical endpoints provides no mechanistic insight that would improve the predictive capabilities of models and could support the development of chemicals that are safe and sustainable by design (SSbD). These themes have emerged as a focal point of authorities and regulatory bodies during the past decades, giving rise to numerous initiatives to modernise the process of chemical safety assessment. This has also initiated a shift from the traditional assessment of predefined endpoints towards mechanistic toxicology.

Mechanistic toxicology supports the evaluation of chemical exposures through the application of the adverse outcome pathway (AOP) framework and toxicogenomics. While AOPs depict mechanisms of chemical exposures as causal chains of interlinked key events (KEs) at various levels of biological organisation, toxicogenomics focuses on elucidating the mechanism of action (MOA) of chemical exposures using omics technologies, such as transcriptomics and methylomics. Investigation of these molecular mechanisms enables a comprehensive characterisation of the effects of chemical exposures while also supporting the development of predictive approaches to chemical safety assessment. Similarly, understanding the MOA of these exposures provides valuable input for the development of chemicals, (nano)materials, and drugs that are SSbD.

Although omics technologies have been widely applied and accepted across the life sciences, their implementation in chemical safety assessment has been hampered by the lack of standardisation and uncertainties in the analysis and interpretation of such data (Pain et al., 2020). This has resulted in concerns regarding reproducibility and challenges in the translation of the complex signatures into meaningful insight and concrete assays to support chemical safety assessment.

In this dissertation, the challenges standing in the way of systematically incorporating toxicogenomics-based evidence into chemical safety assessment are investigated by focusing on three aspects of toxicogenomics data: its intrinsic characteristics, functional properties, and translational potential.

Intrinsic characteristics of data reflect its availability, quality, and FAIRness (findability, accessibility, interoperability, and reusability) (Wilkinson et al., 2016). These characteristics arise from experimental design and technical execution as well as data (pre)processing and reporting. Together, these form the foundation of any model and application of toxicogenomics data. Although data is recognised as one of the most valuable assets in the modern world, it quickly turns meaningless unless its functional properties, i.e., how the data is used, are addressed. This, on the other hand, calls for robust analytical approaches and advanced models that turn bare data into meaningful, functional information. Finally, the full potential of toxicogenomics data is unleashed when it becomes translational, turning data into human-readable format and robust assays for chemical safety assessment that no longer require advanced expertise on computational modelling. Assessing the translational potential of data reintroduces it into the laboratory where new data can be generated in a more targeted manner in the form of data-driven new approach methodologies (NAMs) ready to tackle specific questions relevant for chemical safety assessment.

2 LITERATURE REVIEW

2.1 Brief history of toxicology

The study of toxic substances and their effects on living organisms has evolved over the centuries, driven by a growing understanding of chemical interactions and their potential health effects. The origins of toxicology can be found in ancient civilisations, where early observations on the poisonous properties of certain plants, animals, and minerals were made (Watson and Wexler, 2009). Toxicology as a distinct scientific discipline, however, began to emerge during the Renaissance period. Notably, Paracelsus, a Swiss physician and alchemist in the 16th century, is often regarded as the father of modern toxicology (Langman and Kapur, 2006). He formally introduced the concept of the dose-response relationship, laying the grounds for understanding the fundamental principle that the toxicity of a substance is dependent on its dosage.

Around the same time, occupational hazards associated with mining practices were recognised (Gochfeld, 2005). The rise of occupational diseases and widespread environmental pollution sparked concerns about the adverse effects of industrial chemicals on human health. This led to the establishment of the first toxicological testing methods and the recognition of occupational and environmental health hazards. In the following centuries, toxicology underwent further development and refinement. The introduction of experimental animal models enabled the systematic investigation of the toxic effects of chemicals and the development of standardised testing protocols.

The mid-to-late 20th century witnessed a shift towards a more regulatory-oriented approach in toxicology. Governments and regulatory agencies recognised the need for systematic evaluation of chemical safety pre-emptively, leading to the establishment of toxicological testing guidelines and protocols (Choudhuri et al., 2018). Animal testing became a central paradigm in safety assessment aimed at determining the potential risks of chemicals to human health and the environment. However, the ethical concerns and limitations associated with animal testing

prompted the exploration of alternative methods towards the end of the 20th century.

Today, the development of *in vitro* systems, *in silico* models, and high-throughput screening techniques provide alternative avenues for toxicity testing, with the potential to reduce reliance on animal models. These developments have marked a significant turning point in toxicology, fostering the emergence of new NAMs that prioritise human relevance, efficiency, and ethical considerations – the standpoint of modern chemical safety assessment.

2.2 Current topics in chemical safety assessment

The human pursuit for innovation and constant aim to improve the quality of life has resulted in the discovery and synthesis of the vast number of chemical substances available today. The composition of these substances ranges from simple chemical elements and compounds to alloys and advanced materials, such as engineered nanomaterials (ENMs). Chemical substances continue to be created at an accelerating pace to meet the needs of various industries, including agriculture, pharmaceuticals, electronics, and materials science (Tickner et al., 2021). However, this rapid influx of novel substances has outpaced the capacity of current practices in chemical safety assessment. As a consequence, a substantial portion of chemicals available in the market remains inadequately characterised for their potential risks to human health and the environment. Out of the estimated 100,000 chemicals currently traded in Europe, only approximately 500 have been extensively characterised (European Environment Agency, 2019). This means that they have been evaluated for most known hazards, are regularly quantitatively monitored in most media, and have defined exposure limit values. While additional 30,000 chemicals are characterised for some hazards and may be affected by specific limit values, the remaining 70,000 chemicals are poorly characterised for their potential hazards (European Environment Agency, 2019).

Understanding the challenges in the present framework forces a critical look into the current practices of chemical safety assessment, the process that aims to identify potential hazards, exposure scenarios, and the likelihood of adverse effects from exposure. Chemical safety assessment employs various areas of research and testing, ranging from academia to industries and regulators. Research on chemical safety forms the foundation of the process and gives rise to the methods applied in the regulatory setting. As the methods become robust enough, reaching a strong level of

consensus, standardisation and validation, they can be adopted into the legislation by the regulators (Bas et al., 2021). Hence, the methods and processes in place today have often been developed decades ago, while new methods are being slowly introduced and adopted. This holds true with the REACH (registration, evaluation, authorisation, and restriction of chemicals) legislation that overlooks chemical safety in the European Union. While the intricacies of regulatory chemical safety assessment are beyond this dissertation that focuses on chemical safety research, the considerations made here can also serve as a stepping stone towards broader regulatory acceptance of omics-based evidence.

The current framework mainly operates under the premise of phenotype-based assessment, which focuses on measuring and evaluating phenotypic effects, or apical endpoints, of chemical exposures (Gwinn et al., 2017). Despite major developments in alternative approaches, these assays are still largely based on animal experimentation. The evaluation of apical endpoints of toxicity using animal models has resulted in a slow and high-cost evaluation of individual chemicals one endpoint at a time. At the same time, animal experimentation has been under scrutiny for decades. The 3R principles summarising the replacement, reduction, and refinement of animal use in research were introduced back in 1959 (Russell and Burch, 1959). Today, animal experimentation is not only recognised as an expensive, laborious, and time-consuming activity, but animal-based evidence is not always applicable or easily translated to human health (Landi et al., 2021; Price et al., 2022; Van Norman, 2019). These challenges and limitations have sparked major investments from the regulators, academia, and industry towards the development of alternatives to traditional animal experimentation and beyond the phenotype-based chemical safety assessment.

2.2.1 Phenotype-based assessment

The roots of phenotype-based chemical safety assessment lie in the recognition of observable traits or characteristics as valuable indicators of organism's response to chemical exposure. One of the most notable milestones affecting hazard assessment for human health is the discovery linking genotoxicity and carcinogenesis, consolidated by Ames et al. in 1973, resulting in one of the key test methods still in use (Ames et al., 1973; DeMarini, 2019). This idea of using phenotypic changes as proxies of chemical toxicity pervaded the field of chemical safety assessment, leading to myriad methods and assays in use today.

In animal-based assessment, the hazard associated with chemical exposures is evaluated by exposing the animals to the chemical *via* the most relevant routes of exposure. For instance, in the case of evaluating the hazard associated with inhalation of particulate material, the animals are exposed to the material through inhalation (or other applicable means) and histopathological changes are evaluated (Fraser et al., 2021; Mercer et al., 2013; Porter et al., 2013). Similar principles are applied for various other types of endpoints, including hepatotoxicity, neurotoxicity, endocrine disruption and reproductive toxicity, for example (Pan et al., 2019; Patisaul et al., 2018; Vorhees et al., 2018).

Over the years, the core concept of phenotype-based assessment has translated into *in vitro* assays established to answer the global attempts to implement the 3R principles in chemical safety assessment. Instead of measuring specific endpoints in an animal model, these assays focus on similar proxies on cell or tissue models *in vitro*. At their simplest, these assays include cytotoxicity, cell viability and genotoxicity (Moore et al., 2010). Although *in vitro* cytotoxicity is a widely applied method in chemical safety assessment, it should not be used as a general measurement of the health hazard of a compound. Instead, it can inform on the biocompatibility of chemicals and materials, and it can serve as a tool to screen for appropriate dose ranges for more specific, targeted assays (Li et al., 2015; Ukelis et al., 2008). More advanced alternative models that have also been introduced into regulatory chemical safety assessment include tissue models for skin irritation, skin corrosion, and phototoxicity (Stucki et al., 2022).

The hazard posed by chemical exposure is quantified by modelling the endpoints as a function of the exposure properties, such as the physicochemical properties of the chemical, the dose, and the duration of the exposure. These can be further used to define critical values and limits for exposures, including the no-observed-adverse-effect level (NOAEL) and the lowest-observed-adverse-effect level (LOAEL) (Haber et al., 2018). The challenge, however, is that the endpoint needs to be observed to enable this type of characterisation, necessitating long and laborious experiments for the evaluation of chronic and repeated exposures, for example. Furthermore, focusing merely on the exposure properties and apical endpoints provides little insight into the mechanisms through which chemicals exert their effects, providing a "black-box" assessment of individual apical endpoints with limited mechanistic insight or predictive value.

The chemocentric view where chemical toxicity is defined as a function of chemical properties holds true for the non-testing strategies applied in chemical safety assessment. The characterisation of physicochemical properties enables the

comparison of the chemical with those already in the market, hence facilitating the identification of existing information based on the likeness of the substances. Non-testing strategies, such as chemical grouping, read-across, and quantitative structure-activity relationships, or QSAR for short, use this information to infer or predict potential hazards associated with new or less characterised chemicals (Raunio, 2011).

Read-across is a technique used in chemical safety assessment to predict the potential toxicity of a chemical substance by comparing its properties with those of a similar substance with known toxicity profile (Schultz et al., 2015). The process involves the identification of chemicals that are structurally similar to the target chemical and already have relevant data on their toxicity. This data is then used to assess the potential hazards of the target chemical. QSAR, on the other hand, is a computational technique used to predict the potential toxicity of a chemical substance based on its structural and physicochemical properties. QSAR models are developed using mathematical algorithms and statistical methods that analyse the relationship between chemical properties and toxicological outcomes (Peter et al., 2019).

The use of these computational approaches, however, requires robust data on the known toxicological effects of the compounds. Similarly, careful consideration of the applicability domain of each model is warranted (Klingspohn et al., 2017). This, on the other hand, is primarily restricted by the availability of training data, and such large chemical spaces are rarely available. Hence, substances with little structural similarity to well-characterised chemicals require the generation of new data on their toxicological effects, bringing us back to the assays used to characterise these effects.

These phenotype-based *in vitro* assays and *in silico* approaches define the first generation of alternative assays. While these methods solve some of the challenges associated with animal use, traditional *in vitro* assays remain a simplified model concerned with individual endpoints. Similarly, *in silico* methods, such as read-across and QSAR, rely on robust data from related chemicals, typically assuming the effects are a mere function of the physicochemical properties while omitting the importance of the interaction between the biological system and the exposure. Furthermore, the battery of current alternative methods entails similar challenges as their animal-based counterparts, lacking mechanistic insight that could support early hazard identification and input for the development of the next generation of chemicals that are SSbD. These challenges have resulted in a situation where only a fraction of potential adverse effects is characterised pre-emptively, and many of the detrimental outcomes are uncovered even decades after the initial exposure through

epidemiological investigation (Braun et al., 2016; Dodson and Hammar, 2005; Maffini et al., 2021).

2.2.2 Safety assessment of engineered nanomaterials

ENMs are chemicals with exceptional properties that set them apart from the more traditional chemical substances, such as small molecules. ENMs are manufactured substances comprising minuscule particles at the nanoscale. That is, one or more dimensions of the individual particles measure between 1 and 100 nm. These chemicals, often grouped under the category of advanced materials, have been gaining popularity across various disciplines. Their unique nanoscale properties have sparked myriad applications that now range from bio-medical uses as drug carriers and vaccine components to food additives and ingredients in cosmetics, paints, and coatings (Albalawi et al., 2021; Santos et al., 2015). These advances placed nanotechnology as one of the key enabling technologies in the EU (Páez-Avilés et al., 2018). The rapid growth of the industry is reflected in a surge in nanoenabled products, with the number of such consumer products in the European market having more than quadrupled in the last decade (https://nanodb.dk/en/analysis/consumer-products/#chartHashsection, *last visited in October 2023*). At the same time, the increased applications have sparked concerns related to the potential adverse outcomes associated with ENMs.

Indeed, the same properties that make ENMs attractive for a range of applications also make them unpredictable in terms of hazard for human health and the environment. The high surface to volume ratio increases their reactivity, which on one hand, enables flexible modification of the surface properties, but on the other, it increases their fickle interactions with biological systems (L. Xu et al., 2018). This property together with other implications of the nano-scale size further sets them apart from their bulk counterparts. Similarly, the high reactivity translates to high chance of environmental transformations, such as agglomeration, aggregation, dissolution, redox reactions, and interactions with biomacromolecules (Johnston et al., 2020; Lowry et al., 2012). Hence, ENMs under real-life conditions are rarely comparable to their as-produced-state. Moreover, they are inherently diverse even upon production. This means that the particle size and shape are measured as a range instead of an absolute value, making the final product a mixture of particles of a certain size and shape (Modena et al., 2019). With minor alterations in particle sizes affecting their biological effects, the safety assessment of ENMs under the traditional

toxicology framework has proven complicated (Johnston et al., 2020; Roduner, 2006). Moreover, various ENMs are known to interfere with some of the assays regularly used in chemical safety assessment (Ong et al., 2014).

The recognition of the challenges introduced by the safety assessment of ENMs have placed the nanosafety community at the forefront of the change in current practices of chemical safety assessment. This has been reflected in the financial investments directed towards nanosafety in the EU, giving rise to various projects and initiatives aimed at advancing the safety assessment of ENMs as well as the safe and sustainable design of new, functional ENMs (Afantitis et al., 2020; Furxhi et al., 2023; Mech et al., 2022). Consequently, ENMs are also selected as a relevant topic in this dissertation.

2.3 Paradigm shift in chemical safety assessment

The last two decades have witnessed a global paradigm shift in chemical safety assessment fuelled by technological advances. Efforts such as the U.S. National Toxicology Program (NTP) Tox21 vision have advocated for a transition from *in vivo* animal testing to *in vitro* and *in silico* methods for chemical safety evaluation. The vision was further consolidated by a landmark report on the topic by the U.S. National Academy of Sciences highlighting the need for refining traditional toxicology assays and developing rapid, mechanistic predictive screening methods to inform decision-making for public health (Krewski et al., 2010). The strategy envisioned increased efficiency in safety assessment through *in vitro* toxicity pathway assays using high-throughput screening methods with quantitative parameters.

The aims to reduce reliance on animal experimentation are shared by various initiatives across the globe. Like in the Tox21 vision, the demand for a more profound understanding of toxicity mechanisms has also been recognised in Europe. The new paradigm embraces a more holistic and integrative framework that considers the complex interactions between chemicals, exposure scenarios, and biological systems. This has resulted in major investments towards these efforts. Large-scale research initiatives funded by the European Commission, such as SEURAT-1, EU-ToxRisk, and PARC have advocated for the development and utilisation of NAMs and Integrated Approaches to Testing and Assessment (IATA), often with a highly mechanistic outlook (Gocht et al., 2015; Krebs et al., 2020; Marx-Stoelting et al., 2023). Profound understanding of the interactions between chemicals and biological systems is not only crucial for the development of more

informative and translatable models with better predictive capabilities, but also for the implementation of the SSbD concept.

2.3.1 New Approach Methodologies

NAMs are technologies or methods that focus on the characterisation of chemical hazard and risk without using whole animal models, especially mammalian species (European Chemicals Agency, 2016). They include both existing and novel *in vitro* assays, as well as *in silico* and *in chemico* models and their potential combinations. Despite their name, NAMs are not necessarily newly developed methods per se, but the novelty lies in the application, with NAMs being intended for the replacement of conventional testing strategies to improve the predictability, reliability, and relevance of scientific data to chemical safety assessment (Stucki et al., 2022).

Although still an emerging concept, major investments towards the development, implementation, and acceptance of NAMs has resulted in a rapid surge of scientific reports describing or discussing NAMs. In the last five years, the number of publications mentioning NAMs has steadily grown from less than 10 reports in 2018 to 128 published articles in 2022, according to a Scopus search with the term "New approach methodology" (https://www.scopus.com/). The suggested approaches range from predictive biomarkers to complex high-throughput assays screening large panels of chemicals simultaneously (Fragki et al., 2023; Harrill et al., 2021; Thienpont et al., 2023).

NAMs are increasingly mechanistic and performed in a high-throughput manner. This direction supports the efforts of deeper understanding of the mechanisms through which chemical exposures induce their effects. The mechanistic frame for testing and assessment is often provided by the AOP framework, resulting in NAMs that target the monitoring of specific KEs, further enabling the link between evidence derived through non-animal approaches to systemic, often long-term adverse outcomes. Indeed, the assessment of long-term effects and adverse outcomes of chronic exposures remains one of the largest challenges to overcome without the use of animal experimentation. Although there is still substantial work to be done in this domain, NAMs hold great promise for advancing science while reducing the use of animals in research.

2.3.2 Integrated Approaches to Testing and Assessment

While NAMs refer to the individual tests and methods to generate data and assess specific aspects of chemical exposures, the battery of different methods and forms of evidence can be integrated into a framework referred to as IATA. As the name suggests, IATA integrate various lines of evidence to gain a comprehensive understanding of the hazard and risk that chemical exposures may pose (Sakuratani et al., 2018). They can incorporate evidence derived through traditional approaches as well as through NAMs. In the context of IATA, the use of computational methods can extend to data interpretation and integration (Afantitis et al., 2020). Regardless of the methods used in IATA, their design and application involve expert judgement, and they should be developed to address specific scenarios of toxicological importance.

Like NAMs, IATA are increasingly intertwined with the AOP framework. AOPs often introduce the backbone for the development of IATA, suggesting the types of evidence and methods needed for comprehensive assessment (Tollefsen et al., 2014). Indeed, the power of IATA lays in their capability to leverage existing data and integrate it with newly generated evidence. Combined with AOPs, IATA and NAMs have the potential to bridge the gap between scientific research and regulatory decision-making, leading to better public health outcomes and more sustainable products.

2.3.3 Mechanistic toxicology

The phenotype-based approach to toxicology has been highly focused on modelling chemical hazards as a function of their physicochemical properties with little regard to biological mechanisms. This is not only true in the case of animal experiments, but also the first generation of alternative approaches, such as *in vitro* assays and computational models, including QSAR and read-across. However, rapid developments in biomedical fields combined with technological advances have enabled a deep dive into the molecular mechanisms behind drugs, chemicals, and disease pathology (Manzoni et al., 2018). This information has further leaked into toxicology, giving rise to the distinct branch of mechanistic toxicology, which now focuses on understanding the underlying biological mechanisms by which chemicals or other substances cause adverse effects in living organisms. The long overlooked mechanistic side of chemical exposures is now emerging as an important factor to modernise chemical safety assessment. Mechanistic data on chemical exposures can

support the development of predictive models and hazard identification early in the research and development process, as well as improve interspecies extrapolation, promoting the development of non-animal approaches (Liu et al., 2019). Similarly, profound understanding of chemical-biological interactions and mechanisms is central for adopting the concepts of SSbD (Nymark et al., 2020).

Although mechanistic toxicology is often described as a field focused on molecular mechanisms of toxicity, mechanistic studies can address the processes disrupted by chemical exposures at any level of biological organisation, be it molecular, cellular, or even population level. These mechanisms can be further organised into AOPs, as presented in the Section 2.3.4 dedicated to this crucial concept. Similarly, another fundamental concept falling under the umbrella of mechanistic toxicology is the star of this dissertation, toxicogenomics, which is discussed in the Section 2.4.

2.3.4 Adverse Outcome Pathways

The concept of AOPs presents one of the cornerstones of modern toxicology. AOPs emerged as mechanistic models of ecotoxicological relevance, but soon expanded to human health assessment, and more recently, also to model disease progression and pathogenesis (Ankley et al., 2010; Carusi et al., 2018; Clerbaux et al., 2022b; Wittwehr et al., 2021). AOPs provide a framework to organise mechanistic knowledge into causal sequences of events in a multi-scale fashion. That is, the KEs identified in a process leading to an adverse outcome (AO) are described at various levels of biological organisation and linked together by key event relationships (KERs). The sequence is initiated by a special KE, the molecular initiating event (MIE). A schematic representation of an AOP is depicted in Figure 1.

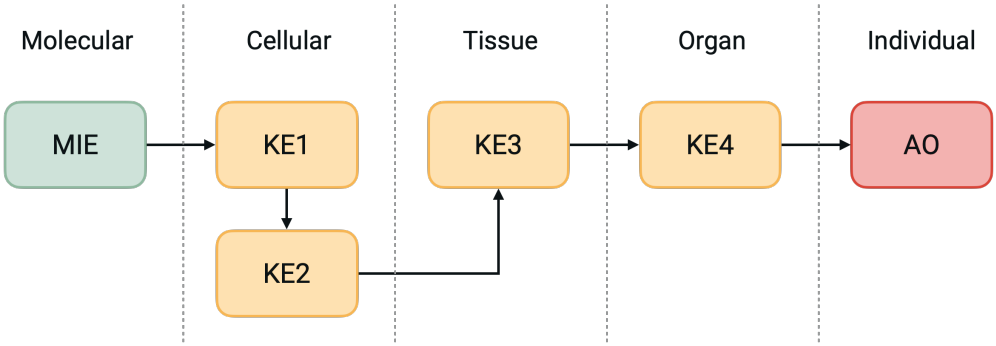

Figure 1. Schematic presentation of an adverse outcome pathway (AOP). Green denotes a molecular initiating event (MIE) while the orange boxes represent key events (KE) in the pathway progressing towards an adverse outcome (AO) at distinct levels of biological organisation. Black arrows correspond to key event relationships (KER).

The AOP framework is overlooked by the Organisation for Economic Co-operation and Development (OECD), and AOP-data is primarily hosted in the OECD AOP Knowledge Base (https://aopkb.oecd.org/, *last visited in November 2023*) and its related AOP-Wiki database (https://aopwiki.org/, *last visited in November 2023*). These resources provide guidance for the development of AOPs and communicate with third party tools to support the use and development of AOPs. Despite the major involvement of regulatory organisations, the development of AOPs is largely driven by individual scientists under the guidelines provided by the OECD. AOPs undergo various stages of development until reaching a point of review and endorsement by the OECD (Svingen et al., 2021).

Some of the main characteristics of AOPs include their inherent modularity and stressor-agnostic nature. The processes are not limited to an individual exposure but rather could be used to support the safety assessment of any exposure able to induce the MIE and KEs. AOPs now serve as a fundamental component of the toxicological knowledge framework that supports mechanistic chemical safety assessment. AOPs play a crucial role in bridging the gap between mechanistic assays and the manifestation of AOs, aligning with the 3R principles by guiding the use and development of NAMs and IATA. In practice, they offer the mechanistic reasoning for chemical safety assessment; what to test and where (Bajard et al., 2023; Patlewicz et al., 2015). This way, individual steps of the AOP can be assessed using NAMs, while the integration of various NAMs with existing information can form IATA. Indeed, one of the most prominent examples of IATA has been reported in the context of skin sensitisation. The IATA is built on an established AOP reporting the mechanistic steps leading to chemical induced skin sensitisation (OECD, 2017).

While this is the framework already employed in the current chemical safety paradigm, AOPs hold the promise to take things even further. AOPs offer the potential to predict multiple AOs with reduced testing by leveraging their network properties (Knapen et al., 2018; Villeneuve et al., 2018). Their modular nature and links between the KEs enable the generation of AOP networks. These networks can be further filtered and refined through careful annotation of KEs that facilitate the generation of subnetworks or the identification of previously unrecognised links (Ravichandran et al., 2022). As the framework matures, AOP networks present exciting opportunities for advanced analytical approaches and models.

The AOP framework is a living organism that keeps evolving. Recent examples have proven the flexibility of the system to expand beyond pathways of toxicological relevance and to accommodate various types of data, including factors that modulate the KEs, further influencing the course of events along the AOP (Clerbaux et al., 2022a; Nymark et al., 2021). Despite the enormous potential of the framework for numerous applications and various stakeholders, success does not come without challenges. The development of new, robust AOPs is a laborious undertaking that is often not considered rewarding for those partaking the task (Carusi et al., 2018). Similarly, AOP-informed NAMs and IATA are developed on a case-to-case basis, as distinct KEs are assessed by individual assays and approaches. In their current format, AOPs provide qualitative associations with little insight to quantitative aspects of exposure. This has been addressed by efforts of developing quantitative AOPs (qAOPs) that introduce quantitative descriptors for the KERs. Although several models have been implemented, their development is challenging and case-dependent due to the complexity of the models (Jeong and Choi, 2022; Jin et al., 2022; Sinitsyn et al., 2022; Spinu et al., 2020). Regardless, these efforts pave the way for future steps of AOP-based toxicity prediction.

2.4 Toxicogenomics

Toxicogenomics emerged as a subdiscipline of toxicology upon the introduction of omics technologies into the field. The term marrying the age-old discipline with modern technology was first mentioned in 1999 in a scientific publication that discussed the introduction of DNA microarrays into the evaluation of toxicity (Nuwaysir et al., 1999). This technological advance allowed the monitoring of the expression levels of thousands of genes simultaneously, unveiling the molecular mechanisms of chemical exposures at the level of gene expression. The value of such

molecular insight could be appreciated due to the lessons learnt in molecular epidemiology. Molecular epidemiology has shown how the profiling of molecular districts in large populations can help build an understanding of the consequences of chemical exposures (Everson and Marsit, 2018; Hoyles et al., 2018; Sun and Hu, 2016; Zeilinger et al., 2013). However, while this a posteriori mechanistic evaluation of chemical exposure is powerful in addressing risk related questions, is not useful when analysing new chemicals or chemicals that are produced in small quantities. To this end, the scientific community has defined premises in the context of hazard assessment that can predict and evaluate consequences of an exposure pre-emptively. In toxicogenomics, omics-based evidence is expected to allow the detection of potential toxicities that may not be observable by conventional approaches, thereby facilitating more accurate and predictive decision-making based on mechanisms of toxicity (Liu et al., 2019). While transcriptomics remains one of the main technologies used in toxicogenomics, the field has expanded to cover the investigation of molecular responses also via other types of omics approaches, including proteomics, lipidomics, and epigenomics, further giving rise to distinct branches, such as toxicoepigenomics (Le Goff et al., 2022; Martins et al., 2019). Given the central role of toxicogenomics in this dissertation, this section will introduce the main technologies applied in toxicogenomics, followed by the current status and future perspectives of the application of toxicogenomics in chemical safety assessment.

2.4.1 Transcriptomics in toxicogenomics

Transcriptomics refers to the study of the transcriptome, the complete set of transcripts expressed in a biological system. It enables the investigation of the response of a biological system to chemical and physical agents on a global scale. Current technologies for genome-wide investigation of gene expression, i.e., the transcriptome, include microarrays and RNA sequencing (RNA-seq). While the popularity of RNA-seq is on the rise due to its rapid developments and the decreasing costs, the two decades of using microarrays has slowed down the transition.

Transcriptomic technologies

Microarrays are a widely applied omics technology, where tiny spots, probes, are embedded on the surface of a chip structure. These probes allow the hybridisation of the complementary sample material that has been labelled with a fluorescent dye. For the assessment of gene expression, the probes are made of DNA and can cover the whole transcriptome of an organism. The RNA extracted from the biological material is reverse transcribed into complementary DNA (cDNA), labelled with a fluorescent dye, and hybridised onto the microarray. Signal intensities are then measured with a laser scanner, and the images are processed to obtain the raw intensities that can be further preprocessed and used for a gene expression analysis.

DNA microarrays have been widely applied in toxicogenomics to investigate the responses of various biological systems to diverse exposures (Igarashi et al., 2015; Kinaret et al., 2021; Saarimäki et al., 2021). As a mature technology, microarrays have the advantage of well-established pipelines and tools for data preprocessing and analysis (Federico et al., 2020). On the other hand, microarrays are limited to the detection of known transcripts due to the probe design. This may also present challenges in highly repetitive genomes given the chance for cross hybridisation (Reilly et al., 2006). Similarly, the technology is prone to batch effects, which need to be recognised and mitigated through careful experimental design and planning to successfully execute the experiments for robust data.

Although DNA microarrays have been the technology of choice for toxicogenomic evaluation, the popularity of sequencing approaches is quickly rising and overtaking microarrays in various fields. The rapid developments in next generation sequencing (NGS) technologies have made it more widely available and attainable. Unlike microarrays, RNA-seq enables *de novo* transcript characterisation and offers a larger dynamic range by omitting the possibility of probe saturation, an issue specific to microarrays (Rao et al., 2018). Similarly, RNA-seq can often provide a more thorough look into the mechanistic aspects of the exposures given its ability to capture long non-coding RNAs often not included in standard microarray platforms (Rao et al., 2018).

At the same time, the long history of microarrays in toxicogenomics means that many of the analytical approaches have been tailored to this type of data (Federico et al., 2020). Regardless of the technology, appropriate data analysis needs to be employed to avoid or mitigate biases in the data. Data generated by RNA-seq is generally more complex and larger in terms of content and size of the generated files, setting demands for the required computational power. Additionally, many of the

reference data sets available for toxicogenomics have been generated with microarrays instead of RNA-seq. Hence, the integration of data derived by the means of different technologies can be challenging (Castillo et al., 2017; van der Kloet et al., 2020). Although the generation of large-scale reference data sets with this technology is still limited by time and costs, more targeted approaches have been established for the screening of chemical exposures in high-throughput manner. For instance, the US Environmental Protection Agency (EPA) has recently initiated systematic screening of various environmentally relevant chemicals using a high-throughput targeted sequencing approach with a panel of approximately 2000 predefined genes as part of the Tox21 initiative (House et al., 2017). These specialised data sets can then be used to define transcriptional points of departure (PODs) for the mechanisms covered by the selected genes.

2.4.2 Epigenomics in toxicogenomics

The focus of toxicogenomics has traditionally been on more transient molecular layers, such as transcriptomics and proteomics, that provide a snapshot of the response at the sampling time. However, the epigenome is emerging as a complementary layer of investigation to thoroughly characterise the molecular events taking place upon exposure (Svoboda et al., 2022). Epigenomics provides the means of explaining the regulatory mechanisms behind gene expression, shedding light also on the potential long-term adverse outcomes of chemical exposures through sustained molecular changes (Ideta-Otsuka et al., 2017).

Understanding the basic concepts of epigenetics is essential to appreciate the role epigenomics can play in chemical safety assessment. Epigenomics itself refers to the study of the epigenome, the complex machinery that controls gene expression. These epigenetic markers are often heritable and modifiable and have the power to influence a phenotype without a change in the actual genotype. Epigenetic modifications include DNA methylation and the changes of the chromatin structure, both at the level of chromatin folding as well as through specific covalent modifications of the histones, i.e., histone modification (Handy et al., 2011). Similarly, regulatory RNAs, such as microRNAs and long non-coding RNAs are considered as part of the epigenetic machinery (Holoch and Moazed, 2015). These epigenetic mechanisms are crucial players in developmental processes and their important role in pathogenesis of various diseases is being uncovered (Skinner, 2011; Zoghbi and Beaudet, 2016).

Epigenetic mechanisms have been investigated as the mediator of the effects of various external factors, including diet, stress, toxins, and pollutants. This has been primed by observations of shared epigenetic alterations between patients with the same disease or phenotype (Baccarelli and Bollati, 2009). Moreover, these observations have resulted in the idea, that the epigenome could serve as a historical footprint of the environmental exposures encountered by an individual. Together, these exposures comprise the exposome that alters the trajectory of health and disease of an individual (Colwell et al., 2023; Wild, 2005). Indeed, epigenetic alterations can affect the health outcomes of individuals across life stages and possibly even across multiple generations (Barouki et al., 2018; Van Cauwenbergh et al., 2020). This way, the role of epigenetic modifications in chemical safety assessment is multiscale. The existing epigenetic state can influence the vulnerability of an individual to new environmental exposures or the epigenetic alterations induced by chemical exposures may directly contribute to the mechanisms of adverse outcomes (Hou et al., 2012). At the same time, deciphering which epigenetic changes are mechanistic mediators of environmental exposures remains one of the main challenges in toxicoepigenomics (Svoboda et al., 2022). Untangling these interactions requires robust mechanistic understanding at various levels of biological organisation, connecting evidence obtained through molecular epidemiology with adequate experimental insight (Bakulski and Fallin, 2014). While examples such as the epigenetic mechanisms of non-genotoxic carcinogens have been investigated as a solution towards better chemical hazard assessment (Desaulniers et al., 2021; Tryndyak, 2017), the potential of epigenomics for chemical safety assessment reaches beyond such specific examples. The potential to employ epigenetic alterations as indicators of long-term hazard without the use of animal models and chronic exposures presents exciting possibilities for the future of chemical safety assessment. Similarly, with the right analytical approach, evaluating the effects chemical exposures may prompt on the epigenome could hold the keys towards models with broader applicability domains due to the highly conserved nature of these molecular markers (del Giudice et al., 2023).

2.4.3 Toxicogenomics in chemical safety assessment

Despite the major advances in omics technologies and the analysis and modelling of omics data, toxicogenomics has not been systematically integrated into chemical safety assessment. The challenges standing in the way of full incorporation of

toxicogenomics-based evidence into the framework has been mainly accounted for the lack of standardisation, concerns of reproducibility, and difficulty of interpreting this complex output (Buesen et al., 2017; Pain et al., 2020). Regardless, toxicogenomics has been extensively employed in the academic setting under the premise that all phenotypic changes are underpinned by molecular changes observable through omics technologies. This has resulted in large collections of data enabling various downstream applications. For instance, a large-scale toxicogenomics database Open TG-GATEs has screened up to 170 chemical compounds in various experimental set ups, while the Comparative Toxicogenomics Database (CTD) has collected over 50 million toxicogenomic relationships from published literature (Davis et al., 2023; Igarashi et al., 2015). These valuable resources have sparked a number of attempts to develop predictive models and other *in silico* approaches to make the most of these data and support development of NAMs and other novel approaches to chemical safety (Aguayo-Orozco et al., 2020; Heusinkveld et al., 2018). Although harmonised large-scale data sets, such as Open TG-GATEs, enable the development of complex models of chemical-biological interactions, the generation of these types of unified data sets is limited by the lack of resources. Hence, the majority of the toxicogenomic data available is derived from independent experiments with varying experimental set ups and platforms challenging their integration.

At its simplest, toxicogenomics characterises the mechanism of action (MOA) of a chemical exposure. The MOA is typically defined as the compendium of molecular alterations observed upon exposure (Kinaret et al., 2017b). Hence, in the case of transcriptomics, the MOA typically refers to the list of differentially expressed genes (DEGs) between the exposed group and the control group. The DEGs can be further characterised functionally through pathway enrichment analysis or other forms of functional characterisation. These approaches have been the foundation of toxicogenomic studies, uncovering the molecular mechanisms of various chemical exposures both *in vivo* and *in vitro* (Kinaret et al., 2021; Labib et al., 2016; Scala et al., 2018; Schyman et al., 2019). The comparison of these signatures in analogous *in vitro* and *in vivo* set ups has further sparked efforts to better extrapolate the results observed *in vitro* to real-life exposure scenarios. Indeed, despite the often reduced complexity of an *in vitro* model, the mechanistic insight introduced by omics has been shown to support *in vitro* to *in vivo* extrapolation (Kinaret et al., 2017b; Liu et al., 2017). Furthermore, the identification of conserved molecular targets and pathways enables the extrapolation of the effects across species. This is not only relevant when extrapolating the results from an animal-based assay to human health, but it is in line

with the emerging One Health approach that extends the consideration to other species, bringing human health risk assessment and environmental toxicology closer together (del Giudice et al., 2023; Saarimäki et al., 2023a).

The mechanistic information derived through toxicogenomics can also support the development of AOPs (Brockmeier et al., 2017). Omics-derived evidence has been used to decipher MIEs and to characterise the pathways altered upon exposure (Chen et al., 2020; Doktorova et al., 2020; Gomes et al., 2019; Jin et al., 2021; Labib et al., 2016). These molecular signatures can be further supplemented with additional data and existing literature to develop robust AOPs. However, the translation of the molecular signatures captured by omics technologies into biologically tangible KEs remains a challenging task that needs to be handled on a case-by-case basis with appropriate experimental evidence.

Similarly, omics data has been explored to derive PODs for genes and biological pathways of interest (Johnson et al., 2022). This approach aligns with the core concept of dose-response familiar in traditional toxicology: the majority of the effects induced by chemical exposures are thought to be dose-dependent. While this is not true for all chemical types, as non-monotonic dose-response relationships have been readily characterised for endocrine disruptors (Hill et al., 2018), the concept forms the basis for the determination of safe exposure limits, such as the benchmark dose (BMD), NOAEL and LOEAL. BMD modelling is considered the state of art method for defining PODs in chemical safety assessment (Haber et al., 2018). This refers to the identification of the dose, at which a significant departure from the steady state can be observed. BMD modelling does this by fitting a mathematical model to experimental data, and the dose corresponding to a pre-defined response (benchmark response, BMR) is identified. The calculation hence takes into account the full dose-response curve, avoiding the challenges associated with NOAEL/LOAEL that rely on the exact tested doses (Haber et al., 2018). This approach has been widely applied to evaluate various toxicological endpoints (Đukić-Ćosić et al., 2022; Farmahin et al., 2019; Vukelić et al., 2023).

In transcriptomics, the genes altered in a monotonic dose-dependent manner are often appreciated as the core mechanism, i.e., the genes directly affected by the exposure, while those showing non-monotonic behaviour are suggested to be affected also by changes in the microenvironment and complex regulatory loops. Hence, BMD modelling has been used to not only identify PODs but also decipher the dose-dependent proportion of the MOA in relation to chemical exposures (Hautanen et al., 2023). This has been facilitated by several tools implemented for

BMD modelling and identification of PODs from omics data (Phillips et al., 2019; Serra et al., 2020b, 2020a; Yang et al., 2007).

Several studies have focused on the comparison of PODs derived through omics and those assessed by the means of phenotypic toxicology (e.g., Li et al., 2023; Thomas et al., 2013). Although these transcriptional PODs are highly dependent on the exposure scenario and selected model system, studies have shown high levels of concordance between gene- and pathway-based PODs and those causing adverse outcomes in long-term studies on whole animal models (Bianchi et al., 2021; Johnson et al., 2020). This opens exciting possibilities for chemical safety assessment as the confidence towards toxicogenomic evidence increases.

Among the many applications of toxicogenomics is the identification of biomarkers of toxicity. In this context, biomarkers are typically (panels of) genes with characteristic expression patterns upon chemical exposures. Hence, these biomarkers can serve as a cost-effective solution to predict the hazard potential of exposures. Although perhaps more prominent in the field of medicine and disease biology, transcriptional biomarkers have been suggested for chemical safety as well. For instance, Fortino et al. identified a set of genes to accurately predict ENM hazard potential both *in vivo* and *in vitro* with the help of multi-layer data (Fortino et al., 2022). Similarly, Samrani et al. suggested candidate biomarkers for developmental toxicology (Samrani et al., 2023). Given the loose definition of a biomarker in the field, the approaches used to identify these potential biomarkers are highly varied. Hence, suggested biomarkers should be carefully validated to evaluate their performance in the intended task. In fact, the first omics-based test method was recently validated and accepted into the battery of OECD approved test methods (OECD, 2023). This test utilises a panel of 196 genes, measuring the expression of these genes to discriminate between skin sensitisers and non-sensitisers (Johansson et al., 2019).

Although many of the examples presented above are focused on transcriptomics, they can be often generalised to other layers of investigation, such as proteomics and metabolomics. Similarly, while the investigation of single omics layers provides meaningful insight to the mechanistic aspects of chemical exposures, their comprehensive characterisation often requires the application of multi-omics, an approach integrating multiple omics technologies. Multi-omics have been applied to a range of biological problems, including the assessment of disease mechanisms and chemical toxicity (Chen et al., 2016; Lake et al., 2018; Lloyd-Price et al., 2019; Quirós et al., 2017; Yachida et al., 2019). Combining multiple omics layers can inform on the interplay of these components, allowing the thorough evaluation of

biological mechanisms leading to AOs of chemical exposures. Various combinations of different omics layers have been used to assess questions of toxicological interest. For instance, transcriptomics has been combined with proteomics to capture the regulatory role of RNA together with the changes translated into proteins (Canzler et al., 2020). Similarly, combining either proteomics or transcriptomics with metabolomics allows the characterisation of the molecular phenotype while also gaining more thorough input on the molecular pathways affected by the exposure (R. Wang et al., 2023; Yen et al., 2023; Yu et al., 2023). From the realms of toxicoepigenomics, DNA methylation has been commonly addressed together with transcriptomics. Changes in DNA methylation have been used to explain the regulatory mechanisms behind transcription and to inform on potential long-term effects of chemical exposures (Szyf, 2011; van Breda et al., 2018).

The high information content of omics data enables complex modelling using advanced computational strategies. The analysis and modelling of omics data has been facilitated by efforts of developing tools and pipelines for conducting these analyses in a standardised and reproducible manner (Marwah et al., 2019; Scala et al., 2019; Serra et al., 2022, 2020b). Although these tools can bring omics data analysis to the fingertips of those with little experience with data analysis, the design, execution, and data interpretation still requires high-level expertise, complicating the large-scale adoption of omics-based evidence in chemical safety assessment. While certain community-accepted standards and best practices are in use, there are no universal rules or standards when it comes to omics data generation, preprocessing, and analysis. Different analyses are often combined in an arbitrary manner and the high content data can result in different interpretations depending on the selected test system, analysis and person doing the interpretation. Hence, there is an urgent need for robust standards and guidelines for toxicogenomics to ensure reproducible pipelines and robust results. Moreover, these challenges highlight the need to translate these experiments into assays that provide easy-to-interpret data relevant for chemical safety assessment.

2.5 FAIR data, high quality data

As the approaches across sciences turn increasingly data-rich, the role of exploiting existing data becomes more and more important. This places emphasis on the aspects of FAIR data. FAIRness is a measure of compliance with the FAIR guiding principles for scientific data management and stewardship (Wilkinson et al., 2016).

These principles were first defined in 2016 to support the **F**indability, **A**ccessibility, **I**nteroperability, and **R**eusability of data in a world of increasing data volume and complexity. Each of the letters comprising the name can be addressed with certain core considerations detailed in the original publication (Wilkinson et al., 2016).

Briefly, findability is supported by the data being registered or indexed in a searchable resource and marked with a unique, persistent identifier. Similarly, the data should be accompanied with sufficient metadata. Accessibility, on the other hand, is enabled by the use of trusted repositories and formats that are both machine and human readable. Interoperability refers to the potential of the data to be integrated and combined with other data sources. This, in turn, is supported by common data structures, and the use of recognised formal terminology and data annotation. Each of these aspects are prerequisites for reusability, which is further supported with clear licensing and data provenance as well as the adherence to community standards, both in data reporting and the experimental design, if applicable.

These generic principles apply to most digital assets, but they can, and should be, supplemented with field-specific recommendations and guidelines. Indeed, the publication of the FAIR principles has been followed by the introduction of various workflows and tools for the evaluation of data FAIRness and FAIRification, as well as discipline specific accommodations of the guiding principles (Cronin et al., 2023; Gaignard et al., 2023; Jacobsen et al., 2020; Nijsse et al., 2022; van Rijn et al., 2022; Welter et al., 2023). These include adaptations of a set of principles for research software and nanosafety data, for example (Barker et al., 2022; Jeliazkova et al., 2021; Papadiamantis et al., 2020).

The aspects of FAIR for omics data are paralleled by specific minimum information guidelines. Namely, these include the Minimum Information About a Microarray Experiment (MIAME) (Brazma et al., 2001) and Minimum Information About a Next-generation Sequencing Experiment (MINSEQE) guidelines (https://www.fged.org/projects/minseqe/, *last visited in November 2023*). Databases intended for sharing omics data, such as the Gene Expression Omnibus hosted by the National Center for Biotechnology Information (GEO, https://www.ncbi.nlm.nih.gov/geo/, *last visited in November 2023*), encourage compliance with these guidelines. Both MIAME and MINSEQE were established before the FAIR guiding principles, but they largely overlap in suggestions. However, MIAME and MINSEQE provide more specific guidance on the required metadata and the preferred data formats for omics data. For example, the importance of describing experimental factors and data processing protocols is underlined.

Similarly, the minimum reporting guidelines emphasise reproducibility and interpretability in addition to the aspects more familiar in FAIR.

The FAIR principles together with other reporting guidelines are pivotal for scientific research and innovation. They enable data to be shared, reused, and combined across different disciplines and sectors. Similarly, increased accessibility and interoperability can also improve the quality and efficiency of research and accelerate scientific discovery. At the same time, it is worth noting that data can strictly adhere to the principles, but still be subpar for its intended application or reuse. FAIRness does not ensure high quality, and the lack of clearly defined community standards and guidelines hampers the consensus on the notions such as "sufficient metadata", as defined in the FAIR guiding principles (Wilkinson et al., 2016). Considerations of the experimental design affecting the data quality and (re)usability through batch effects, for example, are often ignored, and the justification of decisions taken during the experiments and data processing and analysis are not always transparently described.

2.5.1 "Reuse" as the fourth R

FAIR data is not only a key to building better models, but it is also essential for the implementation of the 3Rs in biomedical research, including the field of toxicology. As two of the Rs stand for reduction and replacement, reuse of data is crucial for their implementation. This, in turn, highlights the need for FAIR data.

The reuse of data can directly reduce the number of new experiments performed, including those employing animals. When previously generated data has been adequately reported and shared, the need for generating the same data again decreases (given that the existing data is fit for purpose and of adequate quality). This fosters more sustainable research, decreasing the resources at all stages of the process. Sustainable research and responsible use of resources fuel the ideas of green chemistry and circular economy, further supported by the lessons learnt from existing data (Ncube et al., 2023; Weber et al., 2021). Similarly, the myriad sources of data generated using animals can serve as a reference for the development of novel alternatives. Given the poor availability of human data for toxicity testing, existing data from test methods relying on animals is often required for the evaluation of alternative methods (Griesinger et al., 2016).

At the same time, one of the major challenges in the development of NAMs, especially computational methods, is the lack of adequate data sources (Wu et al.,

2023). Although plenty of toxicologically relevant and interesting data are produced daily, these data are often scattered across databases in various formats. Considering the high demands of data for various analyses and computational models, data curation is often required to obtain such large datasets (Daniel et al., 2022). In this context, data curation refers to the collection of data from various sources. The data can then be harmonised and organised into a format that allows interoperability, and hence, proper reuse of the data. This effort can be facilitated by ensuring the data is FAIR to begin with. It is worth noting, however, that although the data can be curated and harmonised for increased interoperability, the suitability of each individual dataset needs to be evaluated on a case-to-case basis.

Curated data enables various downstream applications, such as meta-analyses that have the potential to unveil novel insights inconspicuous in the individual datasets (del Giudice et al., 2023). Similarly, the discovery of new information can be facilitated by data structures that support data integration and reuse. For instance, knowledge graphs store information in a graph database and represent the relationship between distinct entities, such as genes, chemicals, and endpoints (Pavel et al., 2022a). These entities are depicted as nodes of the network, while the relationships are indicated *via* edges. Further definitions are provided by labels. With robust ontology and well-curated data, knowledge graphs can be used as powerful tools for data analysis and knowledge discovery. For instance, Zhang et al. used knowledge graph embedding to predict adverse drug reactions while Pavel & del Giudice et al. uncovered molecular processes associated with COVID-19 through a knowledge graph infrastructure (Pavel et al., 2021; Zhang et al., 2021).

Given the high information content of omics data, the potential for reuse is enormous. Although defined by the experimental design and its implementation, omics data can be reused and re-analysed for novel insights that might not have been obvious in the original analysis.

3 AIMS OF THE STUDY

In modern toxicology, chemical safety assessment cannot rely only on the measurement of apical endpoints of toxicity. Instead, mechanistic models of the interactions between chemicals and biological systems are pivotal for the study of chemical hazard as well as for the safe and sustainable design of chemicals, including nanomaterials. Toxicogenomics has emerged as a powerful approach for deciphering these interactions. However, toxicogenomics-based evidence is not yet systematically incorporated into chemical safety assessment.

In this dissertation, I aim to investigate how toxicogenomics can bridge the gap between scientific evidence and chemical safety assessment through the implementation of mechanistic models of chemical-biological interactions. The work was divided in specific aims:

1. **Investigating intrinsic characteristics of toxicogenomics data. (Studies I, II, IV)** Intrinsic characteristics of data form the basis for the use of the data. I aim to investigate the intrinsic characteristics of toxicogenomics data that affect its use in chemical safety assessment. Furthermore, I will implement systematic strategies to evaluate and address these characteristics.
2. **Investigating functional properties of toxicogenomic data. (Studies III, IV, V)** The complex signatures derived from omics experiments need to be analysed and modelled for relevant biological insight. Hence, I aim to define novel analytical approaches for toxicogenomics for the generation of robust and informative models of chemical-biological interactions.
3. **Investigating the translational potential of toxicogenomics data (Study V)** Finally, to fully adopt toxicogenomics into chemical safety assessment, toxicogenomics data needs to be translated into robust assays and tangible biological insight that no longer require advanced expertise on computational modelling. Hence, I aim to define systematic approaches to translate molecular and cellular responses into multiscale models and toxicogenomics-based NAMs.

These aims and themes are further depicted in Figure 2.

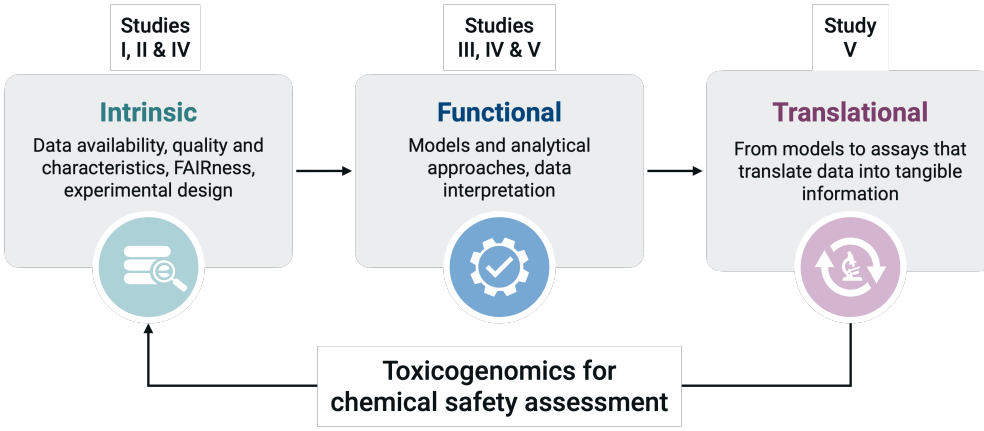

Figure 2. Toxicogenomics data characteristics and properties investigated in this dissertation to support the application of toxicogenomics data in chemical safety assessment.

4 MATERIALS AND METHODS

4.1 Cell work

4.1.1 Cell culture methods

Differentiated THP-1 cells were used as a model of human macrophages in **Study III** and **Study V**. Monocytic THP-1 cells (DSMZ ACC 16) were cultured in cell culture flasks at 37°C using RPMI 1640 media (Gibco, Thermo Fisher Scientific, USA) with 10% inactivated FBS (Gibco), 2 mM ultraglutamine (Gibco), and supplemented with 1% penicillin-streptomycin (Gibco) (complete RPMI media). In **Study III**, cells were plated into six-well plates (1.0 × 10^6 cells/well) and differentiated for 48 h with 50 nM PMA (phorbol 12-myristate 13-acetate, Merck KGaA, Darmstadt, Germany). Fresh, complete RPMI media with PMA was replaced after 24 h and after a total of 48 h, fresh complete media without PMA was added. In **Study V**, cells were seeded in 96-well plates at a density of 1.0 × 10^5 cells/ml and differentiated for 48 h with 25 nM PMA (Merck KGaA). Cells were then left to rest for 24 hours in fresh media without PMA prior to bleomycin exposures.

4.1.2 Chemical exposures and sample preparation

Multi-walled carbon nanotubes (MWCNTs) (**Study III**): A stock solution of MWCNTs at the concentration of 1 mg/ml was freshly prepared in a sterile glass tube using plain RPMI 1640 media (Gibco). The stock solution was vortexed for 1 minute and sonicated 3 x 15 minutes in a bath sonicator (37 kHz, Elmasonic S30 (H), Ilabequipment, USA) at room temperature. The solutions were then diluted into the final subtoxic exposure concentrations (5, 10, and 20 μg of MWCNTs/mL) using complete RPMI media, vortexed and sonicated (additional 15 minutes) again prior to the exposures. Cells grown in complete RPMI were exposed to each of the concentrations for 24, 48, and 72 hours. Control cells were exposed to RPMI media that had been sonicated and vortexed without the MWCNTs to account for any

changes in the media these treatments may induce. All exposures were performed in six replicates to allow the pooling of two samples to obtain the final triplicates used for the RNA/DNA extraction.

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. DNA was isolated from the cell lysates with the Maxwell® RSC instrument and the Maxwell® RSC Cultured Cells DNA Kit (Promega Corporation, USA) following the instructions of the manufacturer. The quality of the RNA was evaluated with Fragment Analyzer (Agilent Technologies, USA) and samples with RNA quality number (RQN) > 9.5 were used for the microarray experiment. Similarly, the DNA integrity was verified by gel electrophoresis in 1% precasted E-gel (Invitrogen, Thermo Fisher Scientific, USA) and further quantified with PicoGreen (Quant-iT Broad-Range dsDNA Assay Kit, Invitrogen).

Bleomycin (**Study V**): Differentiated THP-1 cells were exposed to varying concentrations (2.5, 5, 10, 100 μg/ml) of bleomycin ready-made solution (Sigma-Aldrich, USA) for 6, 24 and 72 hours. The media was then removed, cells washed and lysed, and the lysates from three wells were pooled. Total RNA was extracted with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions and the samples were treated with DNase using the TURBO DNA-free™ Kit (Thermo Fisher Scientific) as per instructed by the provider.

4.1.3 Cell viability

Bleomycin (**Study V**): Cell viability was assessed using the WST-1 method after exposure to 0, 2.5, 5 and 10 μg/ml of bleomycin for 6, 24 or 72 hours. Exposures to 0-100 mg/ml of Triclosan (Sigma-Aldrich) were used as the positive control. 10 μl of cell proliferation reagent WST-1 (Roche) was added to each well and the cells were incubated with it for 3 hours in 37°C and 5% CO2. Absorbance was measured at 450 nm using the Spark microplate reader (Tecan).

4.2 DNA microarray experiments

Gene expression and DNA methylation microarrays were used to study the changes in the transcriptome and methylome of THP-1 macrophages upon MWCNT exposures in **Study III**. The resulting data have been submitted to NCBI GEO

database (https://www.ncbi.nlm.nih.gov/geo/, *last visited in November 2023*) under the series accession number GSE146710.

4.2.1 Gene expression microarrays

For gene expression, 100 ng of total RNA for each sample was amplified with the T7 RNA polymerase amplification method (Low Input Quick Amp Labeling Kit, Agilent Technologies) and labelled with Cy3 or Cy5 fluorescent labels according to the manufacturer's protocol (Agilent Technologies). The labelled cRNA samples were purified using the RNeasy Mini Kit (Qiagen), and the quantity and specific activity of the labelled cRNA was assessed using NanoDrop (ND-2000, Thermo Fisher Scientific). Two samples (300 ng each) labelled with either Cy3 or Cy5 were pooled with each other, fragmented, and hybridised on the Agilent SurePrint G3 Human Gene Expression 8 × 60 microarrays (Agilent Technologies). After hybridisation for 17 hours, the slides were washed according to the instructions of the manufacturer and scanned with Agilent microarray scanner model G2505C (Agilent Technologies). The data were then extracted with the Agilent Feature Extraction software (V12.0.2.2).

4.2.2 DNA methylation microarrays

Genome-wide DNA methylation was assessed using the Infinium HD methylation assay (Illumina, USA) following the manufacturer's protocol. 500 ng of DNA from each sample was bisulfite converted using the EZ-96 Methylation Kit (Zymo Research, USA) according to the standard protocol of the manufacturer. The DNA samples were then amplified, fragmented, and hybridised to the Infinium MethylationEPIC BeadChips (Illumina). Lastly, the microarrays were scanned with the iScan scanner (Illumina).

4.3 Curation of public transcriptomics data

A comprehensive collection of publicly available transcriptomics data from ENM exposures was manually curated in **Study I**.

4.3.1 Data identification and retrieval

A search for relevant data sets was performed on NCBI GEO (https://www.ncbi.nlm.nih.gov/geo/, *last visited November 2023*) and ArrayExpress (https://www.ebi.ac.uk/biostudies/arrayexpress, *last visited in November 2023*) databases with terms "engineered nanomaterial", "nanomaterial", "nanoparticle" and "nanotube". The search was limited to gene expression data by microarray or RNA sequencing, and the organisms specified as *homo sapiens*, *mus musculus*, and *rattus norvegius*. The entries were assessed for the suitability of the experimental set up, selecting those with ENMs as the treatment of interest.

Raw data for microarray-based entries were downloaded from the GEO series entry page, while raw sequencing data in .fastq format were retrieved from the European Nucleotide Archive (ENA) (https://www.ebi.ac.uk/ena/browser/home, *last visited November 2023*). Supporting information (metadata) were obtained from the sample records of GEO using the getGEO function from the R package GEOquery (Davis and Meltzer, 2007). For data available only on ArrayExpress, the metadata were downloaded manually.

4.3.2 Curation of metadata

The downloaded metadata tables were manually curated and unified in R (version 3.5.2). Each table was assigned the following columns in the order as presented: *GSE* (a unique identifier for each data set as defined in GEO)*, GSM* (unique sample identifiers), *treatment* (exposure; ENM or control)*, group* (experimental group; combination of a unique exposure, dose, and time point)*, organism, biological system, dose, dose unit, time point, time point unit, slide, array, dye, platform* and *filenames*. Each of these columns were included in the metadata table regardless of the technology to improve interoperability and reusability. When the variable in the column was not applicable for the specific data set, the column was left unassigned (NA). Lastly, for entries that contained human primary cells, an additional column *donor* was included.

4.3.3 Curation of ENM physicochemical properties

Publications associated with the transcriptomics data sets were identified to manually extract physicochemical characteristics of the ENMs. However, due to the lack of

characterisation and reporting standards, the available information and source of information was highly variable. The publications were used as primary source of information, while additional characteristics were retrieved from the reported supplier information or previous publications using the same material.

The collected characteristics included (where available) the following: ENM name, type, functionalisation and capping/coating, batch/lot information, particle shape, nominal particle size and surface area as determined by transmission electron microscopy (TEM), hydrodynamic size and zeta potential in water and/or in exposure media as well as the potential presence of endotoxin contamination. All information was collected and homogenised into a distinct data table. Missing values were denoted as NA.

4.3.4 Manual quality assessment

The collected data were evaluated for the quality and suitability of the experimental design during and after the initial metadata processing. To reach a unified, FAIR collection of transcriptomics data, a set of data characteristics and quality requirements were defined. These characteristics included 1) availability of raw, non-normalised data, 2) availability of appropriate metadata, 3) minimum of three independent replicates per experimental group, 4) appropriate experimental design (e.g., unmanageable batch effects arising from the lack of dye swapping in two-color microarray experiments would lead to the exclusion of the data set), 5) the platform commercially available and well-represented among the collection (i.e., Agilent, Affymetrix, and Illumina microarrays or Illumina RNA-Seq platforms). Additional quality assessment was performed independently for each data set during the data preprocessing as described in section 4.4.1.

4.4 Preprocessing, analysis, and modelling of omics data

Preprocessing and analysis of both the public (**Study I**) as well as in-house produced omics data (**Study III**) was performed following standard pipelines with state-of-the-art algorithms as defined in (Marwah et al., 2019) and (Serra et al., 2022).

4.4.1 Preprocessing of microarray data

Microarray data were preprocessed each data set at a time by using the R Shiny application eUTOPIA (Marwah et al., 2019) (**Study I** and **Study III**) or a custom script following the same pipeline (**Study I**). The general preprocessing approach for different platforms is presented in Figure 3.

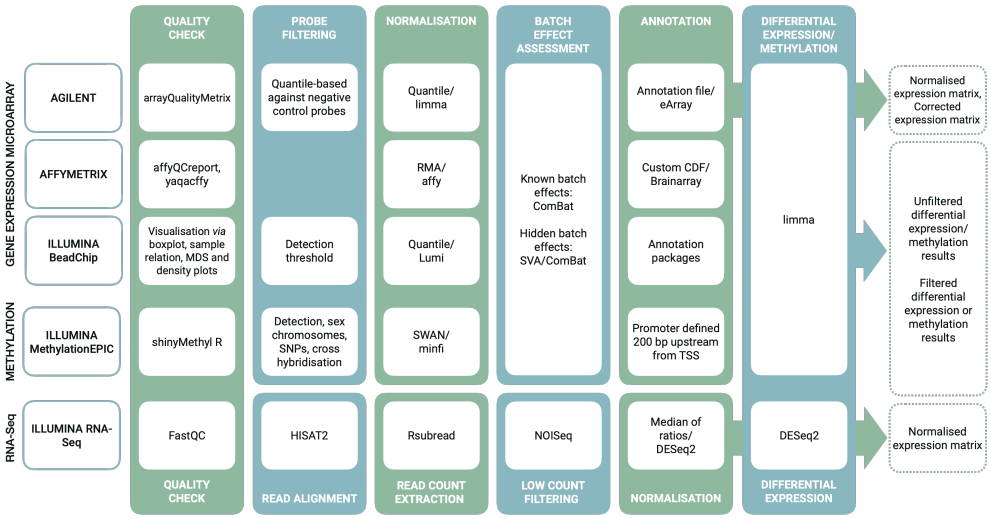

Figure 3. Omics data preprocessing pipelines applied throughout the thesis work. The rows indicate distinct technologies and platform manufacturers (boxes outlined with a solid line) for which optimal methods were selected as indicated in the boxes on the same row. The order of the columns follows the steps applied in the pipeline. Boxes outline with dashed lines indicate the final output obtained from step connected to the box by a thick arrow. Figure modified and updated from **Study I**.

Gene expression microarrays

Briefly, raw data files were uploaded together with the associated metadata. Data quality was evaluated with platform specific approaches as listed in Figure 3. Probe filtering was applied to remove probes with low signal intensities. For Agilent microarrays, a quantile-based approach was used while for Illumina microarrays the probe filtering was based on the detection *p*-values included in the data downloaded from GEO. The data were normalised using quantile normalisation (Agilent, Illumina) or RMA normalisation (Bolstad et al., 2003) (Affymetrix). The presence of batch effects in the data was evaluated through principal component analysis and data visualisation. If batch effects were suspected but no variable(s) reported in the

metadata seemed associated with the batch, surrogate variable analysis was performed with sva (Leek et al., 2012). Identified batches independent from biological variables and variables of interest were adjusted with the ComBat method as implemented in the R package sva (Leek et al., 2012). Probes were then annotated to Ensembl gene or transcript identifiers using the appropriate annotation files or R packages for each platform. For Illumina BeadChips, the organism specific annotation packages (illuminaHumanv3.db, illuminaHumanv4.db, illuminaRatv1.db or illuminaMousev2.db) were used. For Affymetrix arrays, the latest available annotation files for each platform were retrieved from Brainarray (http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/CDF_download.asp, *last visited in November 2023*), while for Agilent, the latest available annotation file for each design was downloaded from the Agilent eArray (https://earray.chem.agilent.com/earray/, *last visited in November 2023*). Multiple probes mapped to the same identifier were summarised by their median values.

Finally, differential expression (DE) analysis was performed using the limma approach (Ritchie et al., 2015) forming contrast pairs between each experimental group (e.g., combination of an independent exposure, time point and dose) and the respective control group. Corrected batches and possible donor information were included as covariates in the analysis. Genes with an absolute log2 fold change (logFC) > 0.58 and a Benjamini & Hochberg adjusted *p*-value < 0.05 were considered significantly differentially expressed.

Given the Agilent annotation files only provided Ensembl transcript identifiers as an option, the data was homogenised after completion of the preprocessing and DE analysis by mapping the transcripts to Ensembl gene identifiers. If multiple transcripts were mapped to the same gene, the one with the highest absolute score, as calculated by $-log(p\text{-}value) \times log_2(fold\ change)$ for each exposure vs. control pairwise comparison, was selected.

DNA methylation microarrays

The preprocessing of the DNA methylation data (**Study III**) was performed with the eUTOPIA application (Marwah et al., 2019). Raw data and metadata were uploaded, and the data were filtered based on the detection *p*-values, removing probes with a detection *p*-value > 0.01 in any sample. Probes for CpGs located on the sex chromosomes, those containing single nucleotide polymorphisms or belonging to a set of known cross-hybridising probes were further filtered out (Chen et al., 2013). The data were then normalised using the SWAN method from the R

Bioconductor package *minfi* (Maksimovic et al., 2012). Technical variation (batch effect) associated with the chip was adjusted by ComBat method as implemented in the R package sva (Leek et al., 2012).

Finally, a gene promoter region was defined as 200 bp upstream from the transcription start site of each gene, and the M-values for CpG probes in the promoter region were summarised by their median value for each gene. These values were then transformed into Beta-values using the function m2beta from the R package lumi (Du et al., 2008) and differential methylation analysis was performed with the limma approach as for the transcriptomics data (Ritchie et al., 2015). Gene promoters with an absolute logFC > 0.26 (corresponding to an absolute fold change of 1.2 as compared to the controls) and $p < 0.01$ were considered significantly differentially methylated.

4.4.2 Preprocessing and analysis of RNA-Seq data

The preprocessing of the RNA-seq data followed a pipeline parallel to the one applied for the microarrays. The steps and methods are summarised in Figure 3.

First, the quality of the data was evaluated using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/, *last visited in November 2023*) and the reads were aligned against the appropriate genome assemblies using the HISAT2 algorithm (Kim et al., 2015). The assemblies used were GRCm38 for mouse data and GRCh38 for human data, respectively. Sequencing file conversions (e.g., SAM files into BAM files) and sorting and extracting uniquely mapped reads were performed using SAMtools (version 1.8-27-g0896262) (Li et al., 2009). Read counts were then computed using the R package Rsubread (Liao et al., 2019) and the human GENCODE annotation (version 35) was applied for human data, while GENCODE version M25 was used for mouse samples. Both annotations were retrieved from the GENCODE project (https://www.gencodegenes.org, *last visited in November 2023*).

Genes with low expression levels were then filtered out from each data set by applying the proportion test as implemented in the R package NOISeq (Tarazona et al., 2015). The data were then normalised using the R package DESeq2 (Love et al., 2014) and the median of ratios method implemented in it. Batch effects were evaluated as in the case of the microarrays, but no significant batches were observed in any of the data sets. Finally, DE analysis was performed with the DESeq2 package following the framework described for the microarray analysis.

4.4.3 Modelling of the dynamic dose-dependent MOA

The dynamic dose-dependent mechanism of action (dMOA) for the MWCNT exposure in THP-1 macrophages was characterised in **Study III**. The dMOA was defined separately for the transcriptome and the methylome following the TinderMIX framework (Serra et al., 2020a). First, sample-wise fold changes were calculated between the exposed samples and their corresponding control samples. These values were then log2 transformed for modelling purposes. In the case of transcriptomics data, two-way ANOVA was first used to identify genes whose fold changes were significantly associated with dose, time, or their interaction ($p < 0.01$). These genes were considered "responsive" and selected for further modelling, while for the methylomics data, all genes were considered.

The fold changes were then used to fit polynomial models (linear, second and third order). The best fitting model was chosen based on the lowest Akaike Information Criterion (AIC) value. Genes with FDR-corrected fitting p-value > 0.05 were filtered out as non-significant.

The dose and time ranges were then distributed to 50 equal bins to produce an activity map for each gene, and a predicted log2 fold change was calculated for each bin based on the optimal selected model for each gene. The resulting activity map hence represents the gene modification (change in transcription and methylation, respectively) as a plane in the space of time and dose with interpolated values for the bins not experimentally evaluated in the study.

The activation maps were then used to identify an area where the expression/methylation status reached the activity threshold (fold change > |1.1|) and was monotonically increasing or decreasing with respect to the dose. This area was denoted as the responsive area. If a responsive area could be recognised from the activation map, the gene was considered part of the dMOA. Finally, the genes were assigned a label (early, middle, or late) by dividing the time axis into three equal sections and evaluating the earliest point at which the gene surpasses the activity threshold with the lowest possible dose.

4.4.4 Functional characterisation of omics data

Study III: Functional characterisation against Reactome pathways (Gillespie et al., 2022) was performed using the R Shiny application FunMappOne (Scala et al., 2019). Lists of genes together with the direction of modification of each gene were

uploaded as input to the tool. The background was defined to include all known genes. Pathways were deemed significantly enriched with an adjusted *p*-value < 0.01 (adjustment with g:SCS method (Reimand et al., 2007)).

4.5 AOP data curation

The AOP framework formed the basis for **Studies IV** and **V**. Annotations already available in the AOP-Wiki repository (https://aopwiki.org, *last visited in November 2023*) were expanded and amended to enable systematic integration of toxicogenomics data and the AOP framework and to support future applications of AOPs. The work was facilitated by large knowledge graph, the unified knowledge space (UKS), previously implemented in the research group (Pavel et al., 2021). First round of KE-gene set annotations was performed with data retrieved in November 2020 and October 2021 (**Study V**). The annotations were further updated with data retrieved in August 2022 and supplemented with the annotation of appropriate biological systems in **Study IV**.

4.5.1 Key event to gene set annotations

The KEs of AOPs considered relevant for human health risk assessment were annotated to curated gene sets, namely, gene sets expressed as gene ontology (GO) biological processes (BP), molecular functions (MF), and cellular components (CC), KEGG pathways and phenotypes, REACTOME pathways, WikiPathways, and Human Phenotype Ontology (HPO) terms. The annotation was performed through a multi-step process comprising techniques of natural language processing (NPL) and manual curation. The pipeline is summarised in Figure 4.

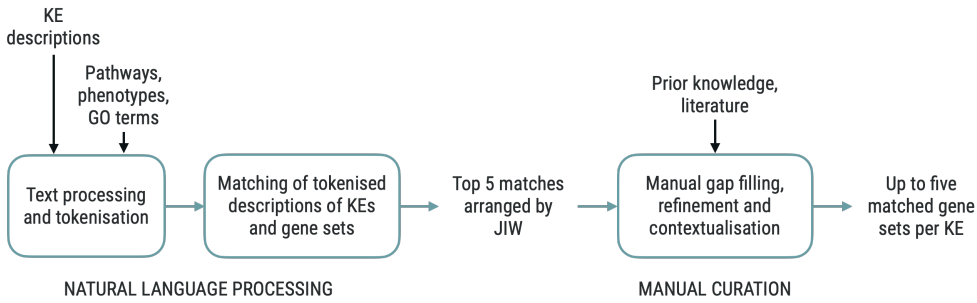

Figure 4. Pipeline applied for the annotation of KEs. The figure was originally published in the supplementary material of **Study V**.

First, NLP was used to prioritise appropriate matches based on semantic similarity. To this end, both the KE descriptions (i.e., names) and the names of the gene set terms in the selected databases were processed and tokenised. The processing included conversion of raw text to lower case only, removal of punctuations and the replacement of multi-word concepts with the same concept expressed in a single word. For instance, the concept "positive regulation" was replaced with the single word "upregulated". The text was then split into tokens so that each token represented an individual word that could be further processed one by one. Tokens corresponding to common stop words, such as prepositions and articles, were dropped. Different declinations of the same concept were converted to their root terms, which meant conversion of plural expressions into singular and the processing of terms such as "increased" and "increasing" into "increase", for instance. Similarly, different expressions and writing styles were standardised by converting symbols into written text.

After the processing steps, each KE description and gene set name was represented by a set of tokens, for example, process "Increased PPAR-alpha activation" would be represented by a set of tokens {upregulate, ppar-alpha}. At this stage, the frequency of individual tokens was considered as the informative value of rare terms was higher than those more commonly used. Hence, the tokens were weighted by their inverse document frequency (IDF), making the weight of the token inversely proportional to the number of gene sets and KEs containing said token. Finally, a weighted Jaccard Index was used to match the KEs and the gene sets by considering the IDF of the tokens in the calculation. Finally, the matches were sorted by the weights in a descending order, prioritising the best matches.

Next, the matches were manually evaluated and refined. The evaluation included considerations of the biological accuracy and context of the match, as well as the refinement of the annotated gene sets to achieve a final set of genes that would most accurately represent each KE. The NLP-based matches were limited to the top five terms, and those deemed inaccurate were removed. The inclusion of up to five gene sets allowed the refinement of the gene set by enabling the inclusion of multiple gene sets, while also keeping the task manageable in terms of the number of annotations.

Manual search across the selected databases, namely WikiPathways (Martens et al., 2021), HPO (Köhler et al., 2021), KEGG (Kanehisa and Goto, 2000), Reactome (Gillespie et al., 2022), and GO (Ashburner et al., 2000), was used to fill the gaps and replace or supplement the NLP-based annotations. KEs describing the alteration of an individual gene were annotated primarily to the main function of the gene (i.e., a pathway driven by the gene) or directly to the individual gene when no distinct pathway could be identified.

Finally, the KEs could be represented by the union of the sets of genes annotated to each KE. Similarly, complete AOPs could be represented by the union of all the gene sets associated to the KEs of the AOP. The genes associated with the annotated terms were retrieved through the UKS knowledge graph (Pavel et al., 2021).

4.5.2 Biological context annotations

The AOP-Wiki repository supports the use of AOP data through various annotations that can be provided by the AOP developers. Among these, level of biological organisation (molecular, cell, tissue, organ, individual, population) is defined and the KEs can be associated with a biological system that indicates the so-called location of the KE. The provided biological systems, however, are often limited to the context of the AOP in which the KE was first introduced, limiting the reuse of KEs, and resulting in unnecessary redundance hampering future applications. Furthermore, these annotations are not homogenised and do not have a full coverage over the KEs. Hence, the existing biological system annotations were manually evaluated and curated to fill the gaps, refine the annotations, and reach a higher coverage and extended applicability of the KEs. Moreover, a hierarchical system that extended to a system-level annotation (i.e., immune system, endocrine system, etc.) was implemented.

The existing annotations and KE descriptions (names) were used as the primary source of information for the task. If the description strictly defined a tissue or cell

type (e.g., "Increase, Cytotoxicity (epithelial cells)"), only the defined biological system was included. In case the existing annotation was limited to a specific biological system, but the process described by the KE was applicable also to other cells, tissues, organs, etc., the annotation was supplemented or replaced with the other possible options. The decisions were based on a literature search to evaluate the possible applicable biological systems. The level of detail in the annotations was based on the KE level; KEs defined at the tissue/organ level were assigned annotations at system and organ/tissue level, while those defined at the molecular level would also receive cell-level annotations, for example.

For KEs of molecular level events, such as changes in gene or protein expression, the Human Protein Atlas (Uhlén et al., 2015) was used to define the relevant cell types and tissues. In case the process was valid for most or all cell types, "eukaryotic cell" was used as the cell annotation, while the system and tissue/organ annotations were left unassigned to indicate the applicability of a range of tissues and organs. In cases where the KE was specified for a distinct cell type or organ/tissue but would be biologically plausible in other cells, tissues, and systems as well, "eukaryotic cell" was introduced as a secondary annotation. This secondary annotation was established to differentiate between any cells of a specific system, organ, or tissue and a generic eukaryotic cell. Finally, the systems, organs, tissues, cell types, and cell components were collected and unified into a dictionary that is provided as part of the data collection.

4.6 Validation of the AOP-gene set curation

The potential of the annotation framework in identifying relevant adverse outcomes from chemical associated gene signatures was evaluated. First, a panel of reference chemicals for hepatotoxicity, carcinogenicity, thyroid hormone disruption, and sex hormone receptor agonism were selected. Hepatotoxic reference chemicals were identified from the European Centre for the Validation of Alternative Methods (ECVAM) reference chemical library (Sund and Deceuninck, 2021), more specifically, from an Excel file listing hepatotoxic chemicals based on EPA Virtual Liver project. The reference chemicals for the remaining endpoints were derived from the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) website hosting panels of reference chemicals for various purposes (https://ntp.niehs.nih.gov/whatwestudy/niceatm/resources-for-test-method-developers/refchem/index.html, *last visited November 2023*). Thyroid

disrupting chemicals were selected from chemicals defined as "ACTIVE" for thyroid activity on a listing based on (Wegner et al., 2016). Similarly, sex hormone disrupters defined as androgen receptor and estrogen receptor agonists were selected from a list of *in vitro* reference chemical provided on the website. These lists were based on previous publications by in (Kleinstreuer et al., 2017) and (Browne et al., 2015), respectively. Lastly, carcinogenic compounds were identified from the 14th report on Carcinogens (RoC classifications), which is available from the NICEATM and lists chemicals that are either known carcinogens or reasonably anticipated to be human carcinogens (RAHC).

The reference chemicals derived from each resource were then matched to the chemicals with reported gene signatures on the comparative toxicogenomics database (CTD) (Davis et al., 2023). This was done based on the provided names and CAS identifiers, retaining only those reference chemicals with a counterpart available on the CTD. The sets of genes associated with each chemical were then obtained via the UKS, and only chemicals with an associated gene set of 50-1,000 genes were considered for the analysis. This criterion was set to minimise the false discovery rate and avoid spurious matches in the enrichment analysis.

An enrichment analysis was performed to evaluate the enrichment of relevant AOPs for each endpoint. Relevance was based on the context of the AOP, i.e., AOPs describing carcinogenicity were considered relevant for the carcinogenic chemicals, etc. To obtain the AOP associated gene sets, the gene sets annotated to each KE of the AOP were combined. The enrichment analysis was based on the Fisher's exact test as implemented in the function enrich from R package bc3net (de Matos Simoes and Emmert-Streib, 2012). AOPs with a false discovery rate (FDR) adjusted *p*-value < 0.01 were considered significantly enriched and the results were ranked from the most significant *p*-value to the least. Lastly, the presence of relevant AOPs among the top 5 enriched AOPs was evaluated.

4.7 AOP fingerprint

The transcriptomics data from MWCNT (Mitsui-7) exposures, both *in vivo* and *in vitro*, were selected from the data collection published in **Study I**. The original datasets can be found under GEO accession number GSE29042 (*in vivo*) and ArrayExpress entry EMTAB6396 (*in vitro*) and the preprocessed data is available in Zenodo as part of the data collection published in **Study I** (https://doi.org/10.5281/zenodo.6425445). The *in vivo* dataset comprised multiple

doses and time points, while the *in vitro* dataset was implemented with a single dose and time point exposure on four different cell lines representing different cell types of the lung. For each distinct comparison (i.e., combination of each dose and time point vs. control *in vivo* and separate cell lines *in vitro*), the DEGs were obtained from Zenodo and filtered by an absolute FC > 1.5 and FRD adjusted *p*-value < 0.05. These DEGs were pooled together to generate the distinct MOA of the exposure *in vivo* and *in vitro*, respectively.

To create the AOP fingerprint for the MWCNT exposures, enrichment analysis was carried out separately against the AOP-associated gene lists and the KE-associated gene lists (KEs mapped to the same gene sets were grouped to avoid multiple testing against the same set of genes), using the Fisher's exact test as implemented in the function *enrich* from R package bc3net (de Matos Simoes and Emmert-Streib, 2012). An AOP was considered significantly enriched if the AOP itself and at least 33% (or a minimum of 2 KEs when the length of the AOP was less than six) of its KEs were enriched with an FDR-corrected *p*-value < 0.05.

4.8 AOP-derived biomarkers

KE-gene set annotations implemented as described in the section 4.5.1. were used as a resource to identify AOP-derived biomarkers for pulmonary fibrosis (PF). First, a panel of characteristics for optimal biomarkers was defined based on the Bradford-Hill criteria of causality used in epidemiology (Hill, 1965). These characteristics are summarised in Table 1. The criteria were then applied to implement a gene prioritisation framework to obtain a list of potential transcriptional *in vitro* biomarkers.

Table 1. Biomarker characteristics defined to guide the prioritisation of genes as potential *in vitro* biomarkers. Table from **Study V**.

Bradford Hill	**Biomarker characteristic**	**Method/Assessment**
Consistency (reproducibility)	Reproducibility	Selection considers evidence from previous profibrotic exposures
Strength (effect size)	Amplitude	Significant alteration of the expression as compared to control
Experiment	Measurable	Transcriptional biomarkers measurable by qPCR; selected genes need to be expressed in the model
Biological gradient (dose-response relationship)	Dose-responsive	Benchmark-dose modelling to evaluate dose-response
Coherence	*In vitro* to *in vivo* extrapolation	Experimental evidence from *in vitro* and *in vivo*[1)]
Analogy	Predictive (of the outcome of interest)	Selection based on the KEs preceding the AO of interest
Specificity	Specificity	Gene ranking based on the specificity score
Plausibility	(Biological) plausibility	The AOP framework provides a plausible context; supporting evidence; selection of the organism
Temporality	Temporality	Transcriptional alteration follows the exposure; selection of the model organism[2)]
–	GLP-method	RT-qPCR
–	Influence	Centrality measures from human protein-protein interaction and gene regulatory networks

[1)]The biomarkers selected here are targeted for the development of non-animal assays for toxicological assessment. Hence the coherence to *in vivo* set ups is not evaluated experimentally. However, *in vivo* data was used for the selection of the markers to provide context of the systemic response. [2)]Temporality in the Bradford Hill criteria refers to a clear distinction of the exposure happening prior to the outcome. Here, temporality was considered by observing transcriptional changes post exposure as well as in the selection of the model organism. Macrophages have a crucial role in the initiation of the profibrotic response preceding the outcome, fibrosis.

4.8.1 Gene prioritisation

The prioritisation of the PF associated genes was based on a multi-step process considering a selection of criteria defined for transcriptional biomarkers as presented in Table 1. The full protocol for prioritisation is summarised in Figure 5.

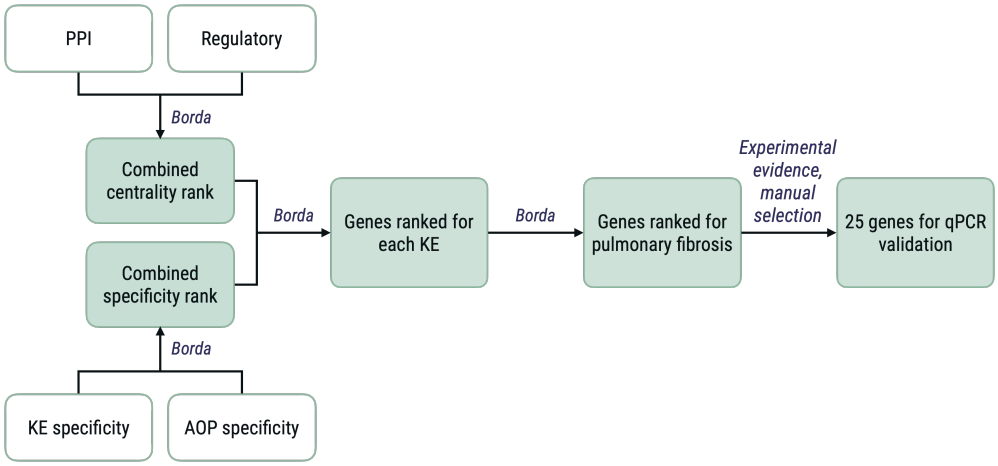

Figure 5. Gene prioritisation pipeline. White boxes with a green outline denote different types of data used to rank the genes initially. Green boxes indicate a ranked list of genes, while black arrows mark the actions performed on the list.

First, the genes associated with any AOP related to PF were ranked by *influence* and *specificity*. For the influence-based rank, various network centrality measures were calculated from the protein-protein interaction (PPI) and transcription factor (TF)-gene interaction data in the UKS as previously defined in Pavel et al. (2021). Briefly, all edges describing human PPI were extracted from the UKS to construct a gene-gene network. As there were multiple data sources with different levels of coverage and sometimes differing information on the PPI, a local threshold was applied on the edges instead of a global one (Pavel et al., 2022b). This mitigates the inherent bias introduced by data availability and popularity of certain gene (products) in research. To this end, the mean number of sources supporting the connecting edges of each node were estimated. The mean number of sources was then used as the minimum number of required sources, and only the edges fulfilling this criterion were included. This action was applied to each node of the type "GENE" as long as the gene was flagged as protein coding in Ensembl (Martin et al., 2023).

The so formed gene-gene network of human genes consisted of 20,260 nodes and 806,250 edges with a network density of 0.0039. The regulatory network (TF-gene), on the other hand, was based on a lower number of data sources and hence each data source was retained and scored equally. These edges were then used to generate a directed gene-gene network which comprised 18,754 nodes, 363,649 edges and a density of 0.001. These two networks were then used to calculate the degree, betweenness, eigenvector, and closeness centrality measures for each node with NetworkX (Hagberg et al., 2008). The measures were then used to rank the PF-

related genes in the context of each individual KE by ranking them from the most to the least central according to each measure. The ranks from different measures were then combined by the Borda method using the function *Borda* from the R package TopKLists (Schimek et al., 2015).

Next, the genes were ranked by a specificity score by KEs and AOPs. The score for KEs was defined as the occurrence of the gene in the KEs belonging to any of the six PF AOPs divided by the occurrence of the gene in any other KE. Similarly, the AOP specificity was defined as a score calculated by dividing the occurrence of the gene in PF AOPs divided by their occurrence in any other AOPs. These scores were then used to rank the genes from more specific to least specific, i.e., prioritising the genes that occurred in PF associated KEs and AOPs more as compared to the rest of the AOPs/KEs. These ranks were combined into a single specificity rank for each KE by the Borda approach as described above. A consensus rank for each KE was then established by combining the influence-based rank with the specificity ranks using another round of the Borda method. Although these KE-specific ranks could inform the selection of KE-specific biomarkers, a final round of Borda was established to obtain a single ranked list of genes associated to PF by combining the ranks of individual KEs. This way, genes ranking high in multiple PF KEs could be prioritised over those more specific to individual KEs.

The final rank based on specificity and influence (centrality) was complemented with experimental evidence which guided the manual selection of the candidate genes from the rank. The experimental evidence included differential expression data from both *in vivo* and *in vitro* experiments with a known profibrotic exposure (Mitsui-7). The *in vivo* data were selected from the data collection in **Study I** (GEO series GSE29042, originally published in (Dymacek and Guo, 2011)), while the *in vitro* data from **Study III** was used as its *in vitro* counterpart (see Section 4.1.2 for details). Particularly, the data were selected so that the experimental set up comprised multiple time points and doses to enable dose-response modelling. The DEGs were retrieved from **Studies I** and **III**. Genes responding to the exposure in a dose-dependent manner at any given time point were identified by the means of benchmark dose (BMD) modelling using the R shiny application BMDx (Serra et al., 2020b). Briefly, linear, second order polynomial, hill, power, and exponential models were fitted for each gene in both data sets, and the optimal model for each gene was selected based on the lowest AIC. The lack-of-fit *p*-values were used to filter the genes by removing those with an optimal model having a lack-of-fit *p*-value < 0.1. BMD values and their lower and upper bounds (BMDL and BMDU, respectively) were then estimated assuming constant variance and by using a benchmark response

factor of 1.349, which is considered to correspond to a minimum of 10% difference as compared to the control samples (Thomas et al., 2007). The remaining genes were filtered by removing those with BMD or BMDU values higher than the highest exposure dose (i.e., 20 mg/ml in vitro and 80 μg in vivo, respectively). The genes remaining after the applied filters were considered dose-dependent and used for the manual evaluation of the gene rank. Genes from the *in vivo* data set were converted to their human orthologs with Ensembl (Martin et al., 2023).

Finally, the feasibility of observing changes in the gene expression in the selected model the gene in the selected macrophage model was considered, as the ranked list was not cell/tissue specific but included all genes relevant for LF under the annotated AOP framework. For example, genes coding for collagen proteins were ranked high but are likely not deregulated in macrophages. Moreover, the coverage over the KEs was included in the collection, which resulted in the selection of genes that might not rank high in the final combined rank but were specific to an individual KE and ranked high in the context of that event. These considerations were summarised in the following list of priority for the manual gene selection over the ranked list: 1) genes deregulated *in vivo* and *in vitro*, with most emphasis on dose-dependency; 2) genes deregulated *in vitro*, with most emphasis on dose-dependency; and 3) genes not significantly differentially expressed but dose-dependently altered. The final selection of candidate biomarkers was supplemented with additional genes that had a lower rank but were specific to KEs that would have otherwise not been covered by the selection.

4.8.2 Biomarker validation with RT-qPCR

Reverse transcription quantitative PCR (RT-qPCR) was used to assess the expression of selected candidate biomarkers in **Study V**. RNA samples from the THP-1 macrophages exposed to varying concentrations of bleomycin for 6, 24 or 72 hours were used for the analysis (see Section 4.1.2 for exposure details).

Using 100 ng of RNA, cDNA was synthetised with the High-capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) following the manufacturer's instructions. CFX96 Touch Real-Time PCR Detection System (BioRad) was used to determine the expression levels of the candidate genes. The reactions were prepared with 10 μl of iO Multiplex Powermix (Bio-Rad, #1725849), 5 μl of the prepared cDNA diluted 5-fold, and 2.5 μl of nuclease-free water (ThermoFisher, #AM9930).

PrimePCR Probe Assays (BioRad) were added (1 µl assay + 1.5 µl nuclease-free water for single assay or 0.5 µl of each assay in a multiplex reaction) according to Table 2.

Table 2. Candidate biomarker genes and their information for RT-qPCR assay.

Gene	PrimePCR UniqueAssayID	Type	Reaction
ACTB	qHsaCEP0036280	Reference	1
SMAD7	qHsaCEP0050142	Candidate gene	2
MMP9	qHsaCIP0028098	Candidate gene	2
GDF15	qHsaCEP0051579	Candidate gene	2
CTSK	qHsaCIP0030907	Candidate gene	2
PLOD2	qHsaCEP0052848	Candidate gene	2
CXCL2	qHsaCEP0058163	Candidate gene	3
LTBP4	qHsaCEP0024931	Candidate gene	3
TGFB3	qHsaCEP0058244	Candidate gene	3
RCN3	qHsaCEP0057804	Candidate gene	3
MMP7	qHsaCEP0052037	Candidate gene	3
SPP1	qHsaCEP0058179	Candidate gene	4
FN1	qHsaCEP0050873	Candidate gene	4
LTBP3	qHsaCEP0053782	Candidate gene	4
RSAD2	qHsaCIP0031596	Candidate gene	4
CCL7	qHsaCEP0058033	Candidate gene	4
IL8	qHsaCEP0053894	Candidate gene	5
MMP19	qHsaCEP0051244	Candidate gene	5
TWIST1	qHsaCEP0051221	Candidate gene	5
PLK3	qHsaCIP0027687	Candidate gene	5
CXCL10	qHsaCEP0053880	Candidate gene	5
LOX	qHsaCEP0050731	Candidate gene	6
PTX3	qHsaCEP0033071	Candidate gene	6
TGFBI	qHsaCEP0058394	Candidate gene	6
CCL2	qHsaCIP0028103	Candidate gene	6
TGFB1	qHsaCIP0030973	Candidate gene	6

RT-qPCR data were analysed by calculating fold change (FC) values using the comparative CT($2^{-(ddCt)}$) method (Livak and Schmittgen, 2001). Log2 transformed FC values were used for outlier detection by removing samples with log2(FC) above the 75th or below the 25th percentiles of the distribution. Finally, the statistical significance of the observed expression patterns was evaluated using ANOVA and tukey HSD posthoc test and the dose-dependency of the candidate biomarkers was evaluated with BMD modelling following the same steps as with microarray data in Section 4.8.1.

5 SUMMARY OF RESULTS

The aim of this thesis was to investigate how toxicogenomics can support chemical safety assessment through the generation of more informative models of biological-chemical interactions. Toxicogenomics has the potential to transform chemical safety assessment from an observational science to an integrative field. The shift in focus from apical endpoints towards profound characterisation of mechanisms of toxicity forms the basis of modern chemical safety assessment, providing tools for predictive toxicology and the design of new chemicals that are not only functional but also SSbD.

The investigation was focused on three pillars of toxicogenomics: the data (**Studies I, II** and **IV**), the models (**Studies III** and **V**), and the assays based on those models (**Study V**).

5.1 Intrinsic characteristics of data

Intrinsic characteristics of data form the basis of any subsequent use of the data. They arise from the combination of the experimental design and technical execution as well as data reporting and its presentation. Hence, the intrinsic characteristics of data reflect its FAIRness, as well as its availability, quality, and overall usability.

In this dissertation, the intrinsic characteristics of data were investigated through a systematic curation of publicly available transcriptomics data from ENM exposures in relevant in vitro and in vivo models (**Study I**), as well as through the curation and annotation of AOP-related data (**Study IV**). These efforts not only resulted in a FAIRified collection of transcriptomics data ready to reuse, but also a robust framework to curate and evaluate toxicogenomic data and annotate AOPs. Moreover, this provided an approach for the systematic integration of toxicogenomics data into the AOP framework.

5.1.1 Overview of the toxicogenomics data collection

In **Study I**, the data collection was curated using a multi-step approach where the initial data search resulted in the identification of 124 potential data entries. Out of these, 84 entries passed the manual quality assessment evaluating the suitability of the data for the collection. These 84 entries were further divided into 101 specific data sets, as those entries comprising multiple biological systems were divided into individual data sets during the preprocessing. The final collection represents various experimental set ups that were categorised into four classes suitable for different modelling approaches:

- Class I – Multiple doses, multiple time points
- Class II – Multiple doses, one time point
- Class III – One dose, multiple time points
- Class IV – One dose, one time point

The division of the data into the defined classes is visualised in Figure 6A. The data was further characterised by the experiment type (*in vivo* vs. *in vitro*) and organism, biological system as well as the core material/material type used in the exposure. The characteristics of the final data collection are summarised in Figure 6.

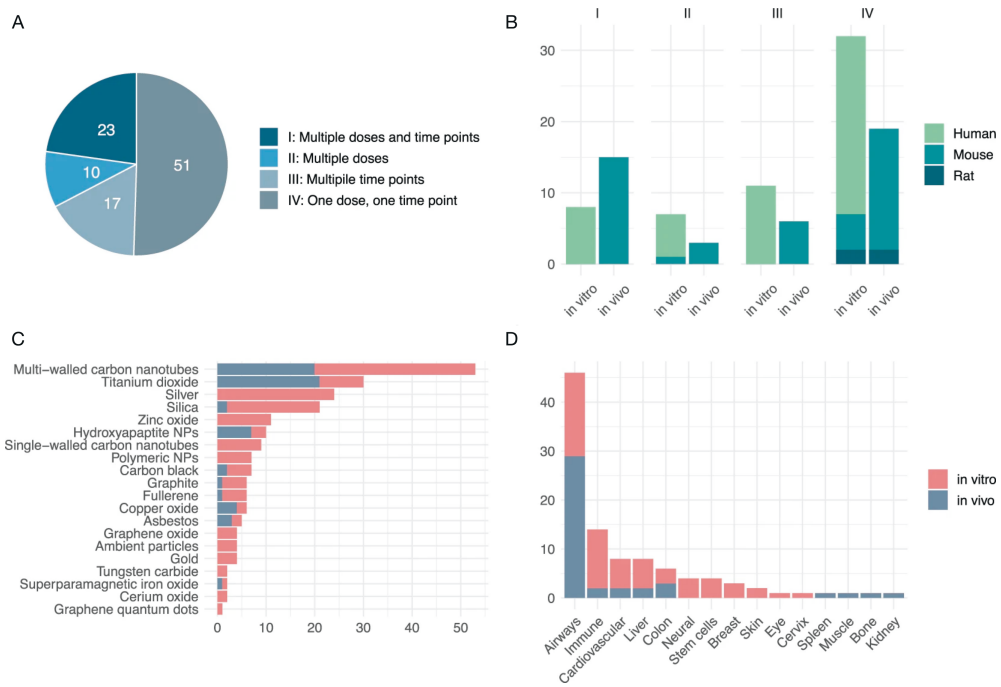

Figure 6. Data characteristics of the curated toxicogenomics data collection. A. Pie chart indicating the number of datasets in each category defined by the experimental design. B. Stacked bar plot representing the number of datasets for each organism divided into *in vitro* and i*n vivo* experiments. C. Stacked bar plot representing different core materials of the ENMs available in the collection with *in vitro* and *in vivo* experiments separated. D. Stacked bar plot indicating the number of experiments by biological system with cells and tissues from the same system grouped under a general category. *In vivo* and *in vitro* exposures separated. Figure originally published in **Study I**.

5.1.2 Data quality and FAIRness as intrinsic characteristics

Considering the aim of curating a unified collection of FAIR data for toxicogenomic evaluation and modelling of ENM exposures, the data identified in the first step of **Study I** went through rigorous quality assessment and investigation as described in Section 4.3.4. This offered a deep dive into the published material enabling the investigation of data properties that may have contributed to the challenges of reproducibility recognised in relation to toxicogenomics.

The data curation revealed systematic gaps in the quality of published transcriptomics data on ENM exposures. The manual quality assessment performed on the initial data entries resulted in the discarding of 40 entries. A total of 26 entries were discarded due to the lack of replicates (consistently less than three independent

replicates per group), while five entries were left out of the collection due to a platform that was outside the scope of the collection (only commercial platforms produced by Affymetrix, Illumina or Agilent were included). Four entries using a microarray design with a two-colour set up were discarded due to them being generated without dye swapping, i.e., the control samples and the treated samples were consistently labelled with distinct dyes. This type of experimental set up does not allow the distinction of technical variation arising from the dye used from labelling from that resulting from the treatment of interest, preventing any meaningful analysis of the data. Two entries found had no raw data available, while two lacked metadata to allow proper preprocessing. Lastly, one entry lacked control samples completely.

5.1.3 Overview of the AOP annotation

AOPs provide the scaffold for mechanistic toxicology, depicting KEs of a toxicity pathway as a causally linked sequence of events. It was anticipated that rigorous curation of molecular events associated to KEs and AOPs would support the usability of the AOP framework for novel applications while also providing the much-needed link between toxicogenomics and relevant biological events at various levels of biological organisation. To this end, the KEs of all human relevant AOPs were associated with established gene sets (terms), such as pathways, gene ontology terms and human phenotype ontology terms.

Originally 231 AOPs comprising 997 unique KEs were deemed human-relevant and included in the annotation effort. Together, these formed 1,636 AOP-KE pairs as the same KEs can be associated with multiple AOPs. As a result of the annotation, 969 unique KEs were associated with at least one term. The number of terms assigned to the KEs ranged from 0 to 5 with a median of 3.

Different term sources were represented in distinct proportions among the annotations (Table 3), with GO biological processes being the most prevalent type with 1,532 instances. GO molecular function terms were present in 273 instances while Human Phenotype Ontology terms were assigned 263 times. These were followed by Reactome pathways (195), WikiPathways (167), KEGG pathways (154), individual genes (89), and GO cellular components (83). These numbers in relation to the total number of KEs expressed at the level of biological organisation are visualised in Figure 7A.

Table 3. Number of unique terms (gene sets) and genes used from each data source together with the total number of human-relevant terms and genes present in each resource. The numbers in brackets express the unique instances of the terms. The number of genes correspond to unique Ensembl gene identifiers.

Data source	Terms included	Total terms	Genes included	Total genes
GO biological process	1,532 (746)	12,380	8,817	20,411
GO molecular function	273 (158)	4,434	5,252	20,878
HPO	263 (171)	9,946	4,233	5,209
Reactome	195 (108)	2,496	6,894	12,355
WikiPathways	167 (69)	701	3,108	8,808
KEGG	154 (61)	334	4,097	9,454
GO cellular component	83 (49)	1,754	8,434	21,809

After annotation, the KEs can be presented as the union of all the genes linked to the gene sets assigned to it. This way, the inclusion of up to five individual terms for each KE enabled the final KE associated gene set to be tailored for specificity. Similarly, this facilitated the implementation of the hierarchical structures present in the original databases, such as GO. For instance, KE 1457 titled "Induction, Epithelial Mesenchymal Transition" was assigned the following terms according to the hierarchy present in GO: GO:0001837 - Epithelial to mesenchymal transition, GO:0010717 - Regulation of epithelial to mesenchymal transition, and GO:0010718 - Positive regulation of epithelial to mesenchymal transition, resulting in a robust set of genes for each KE.

The number of genes associated with the KEs hence ranged from 1 to 6,047 with a median of 82. Similarly, when AOPs are presented as a union of all the genes associated with their KEs, the number of genes by AOP ranged from 15 to 6,381 with a median of 804. In total, the genes associated with the KEs cover 16,825 genes. Some of these genes are specific to individual KE, while others are more generic and associated with up to 234 KEs (Figure 7B).

While the KE-gene set annotations link toxicogenomics into the AOP framework, the usability of AOPs can be supported by various other types of annotations. Here, the focus was on consolidating the biological context annotations already established in the AOP-Wiki. The manual evaluation and assessment of these resulted in the introduction of a hierarchy that supports the use of these annotations as well as the consolidation and gap filling of the existing ones. The hierarchical structure was established by adding a system-level annotation (e.g., respiratory system, digestive system, etc.) and organising the tissue/organ and cell level

annotations under these systems. In total, the biological system annotations cover 18 biological systems or equivalent terms, 86 specific organs and tissues, and 70 cell types. The total number of KEs by biological system as well as the number of distinct cell types and tissues/organs under each biological system are summarised in Figure 7C-D.

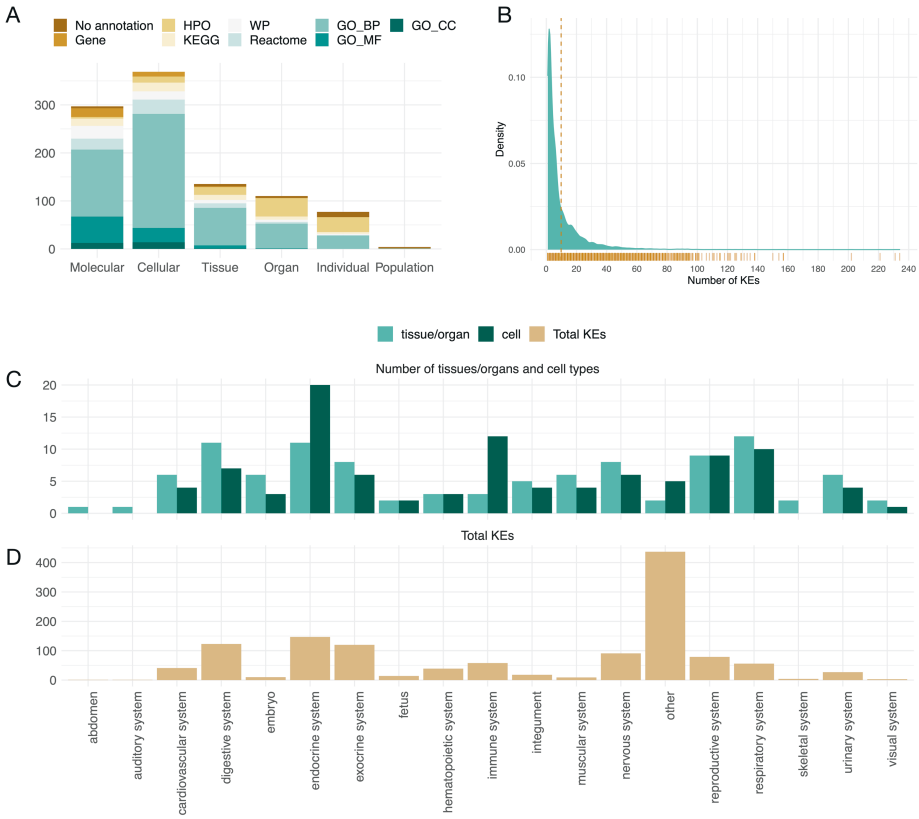

Figure 7. Overview of the AOP annotations. A. Stacked bar plots depicting the proportion of annotation types divided by the level of the KEs. The height of the bar reflects the number of KEs in each group. Dark brown (no annotation) stack corresponds to the number of KEs with no associated gene sets, while the different annotation types are represented proportionally to their use in each level. Abbreviations as follows: HPO = Human Phenotype Ontology, WP = WikiPathways, GO_BP = Gene Ontology Biological Process, GO_CC = Gene Ontology Cellular Component, GO_MF = Gene Ontology Molecular Function. B. Density plot describing the number of KEs associated with each gene. The dashed line indicates the median of the distribution. C. Bar plots representing the number of different tissues/organs (turquoise) and cell types (dark green) mapped to each system-level annotation (D). D. Total number of KEs by system level annotation. The system

"other" includes KEs assigned a cell type applicable for a range of tissues and/or systems, and those for which no system could be defined. Figure originally published in **Study IV**.

5.2 Functional properties of data

Functional properties of data refer to the way the data can be used and what type of information can be derived from the use of the data. In the context of toxicogenomics, functional properties are hence defined as the models enabled by the data and the information generated through that model. Although models of the MOA have been implemented, the typical focus on transcriptomic responses at individual time points and doses provides little insight to the dynamics of the response, only providing a snapshot of the response. Similarly, *in vitro* experiments characterising the MOA are often focused on acute effects and struggle to inform on mechanisms of long-term adaptation.

In **Study III**, the functional properties of toxicogenomics data were investigated by implementing a model of biological-chemical interactions that could support the deduction of mechanisms of long-term effects in a relatively short-term *in vitro* exposure. In **Study V**, on the other hand, the functional properties were assessed through the evaluation of the AOP-based analysis of toxicogenomics data, expanding the horizons of toxicogenomics data analysis and interpretation.

5.2.1 dMOA highlights a distinct set of molecular alterations

In **Study III**, a dynamic dose-dependent mechanism of action (dMOA) of a MWCNT exposure in THP-1 macrophages was defined as the set of genes whose expression (transcription) or promoter methylation (DNA methylation) was altered in a dose-dependent manner during the 72 hours of exposure. This approach enabled a detailed investigation of the dynamics of the molecular alteration while also providing a complementary look into the dose-dependent alterations, aligned with core concepts of dose-dependency in toxicology.

First, the dMOA was compared with the more traditional approach for defining the MOA at the level of the transcriptome and methylome as the sets of differentially expressed genes (DEGs) and differentially methylated gene promoters (DMPs),

respectively. To this end, DEGs and DMPs were defined independently for each dose and time point combination. The number of DEGs showed a dose-dependent trend at all time points, while a similar trend can only be appreciated at 24 hours for the number of DMPs (Figure 8A-B).

The DEGs and DMPs were then compared with the number of dynamic dose-dependent genes and gene promoters by gathering the DEGs and DMPs at independent timepoints and doses together and evaluating the overlap between the sets of genes. This analysis showed that the distinct approaches highlight divergent sets of genes across both molecular layers (Figure 8C-D). Specifically, 58% of the DEGs were also dose-dependently altered, while only 23% of the DMPs were dose-dependent. In both molecular layers, the investigation of the dMOA highlighted a larger number of molecular changes as compared to the traditional approach based on differential expression/methylation. Overall, the effects of the exposure on the transcriptome were found to be more impactful as measured by the number of altered genes/gene promoters.

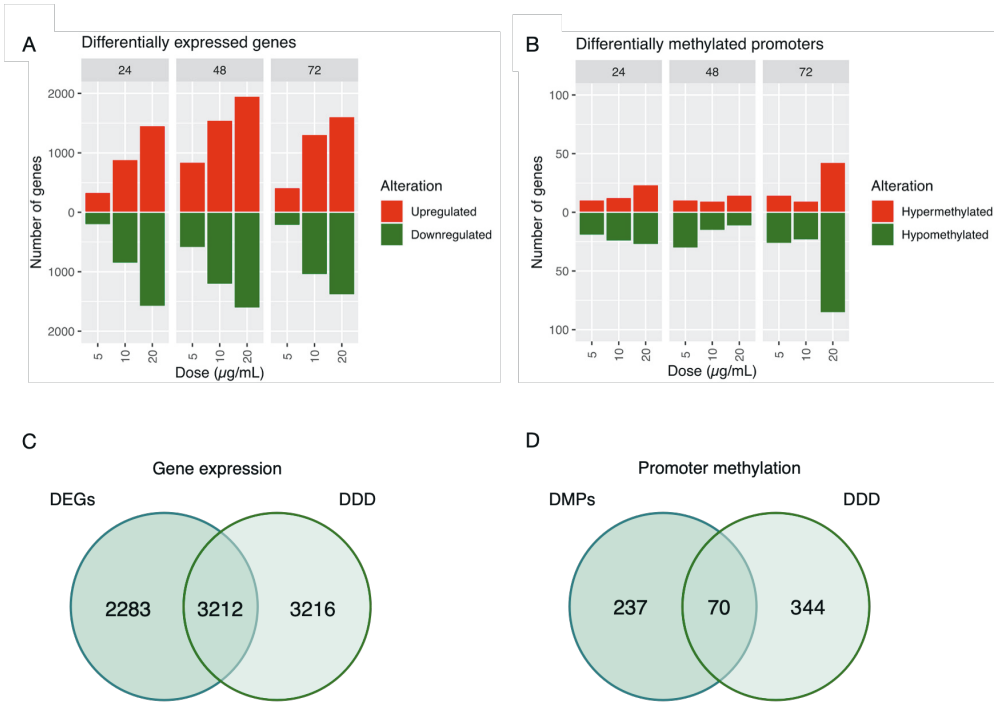

Figure 8. Comparison of the differential expression and promoter methylation with the set of dynamic dose-dependent genes and promoters. A-B. Bar plots displaying the number of differentially expressed genes (A) or differentially methylated promoters (B) between cells treated with Mitsui-7 and control cells. Upwards bars (red) indicate the number of

upregulated genes or hypermethylated promoters at each time point and dose, while downwards (green) bars correspond to the number of downregulated genes or hypomethylated promoters. C-D. Venn diagrams describing the overlap between the sets of differentially expressed genes and dynamic dose-dependent genes (C) or the sets of differentially methylated promoters and dynamic dose-dependent promoters. Figure adapted from **Study III**.

5.2.2 Gene expression and promoter methylation show distinct kinetics of adaptation

In **Study III**, changes in DNA methylation were investigated to further explain the regulatory mechanisms behind macrophage responses to MWCNT exposures and to retrieve relevant information on the potential long-term effects resulting from such exposures. This investigation was based on the premise that DNA methylation at the gene promoter is an important regulator of gene expression, with methylation also being a more stable form of molecular alterations over transcription (Scala et al., 2018). Hence, investigating the interplay of the two molecular layers is important.

In total, the dMOA between transcription and promoter methylation shared 220 genes out of the total of 6428 in expression and 414 in DNA methylation (Figure 9A). The genes were further categorised into early, middle, or late responders based on the kinetics of the molecular changes. The largest overlap between the molecular alterations was observed between early genes in transcription and late responders in promoter methylation (Figure 9B).

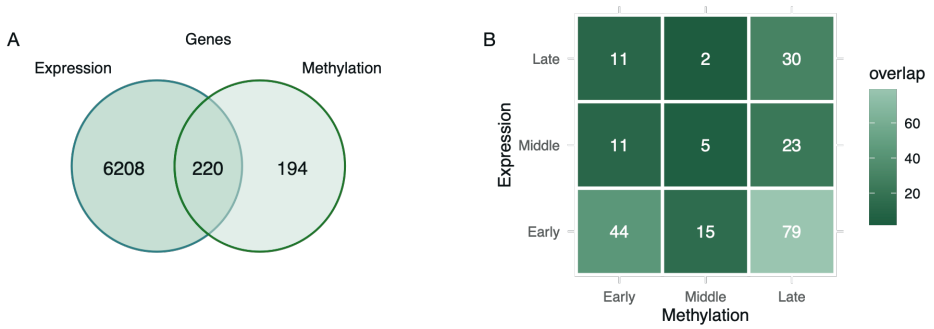

Figure 9. Integration of the dMOA over expression and methylation. A. Venn diagram depicting the overlap between DDD genes in transcription and methylation. B. Tile plot indicating the intersections of genes between each activation group in expression and methylation. Figure adapted from **Study III**.

The distinct kinetics of the two molecular layers were also observed at the level of enriched pathways. 63 enriched pathways were shared between the transcriptome and promoter methylation (Figure 10). The kinetics at the level of the pathways followed the trend observed for individual genes as nearly all the shared pathways were enriched by the early transcriptional responders and late methylation responders. While the enrichment analysis of the transcriptomic dMOA often showed sustained enrichment of the same pathways across the early, middle, and late responders with 63% of the pathways enriched by early responders also being enriched by middle and/or late responders, the enrichment was restricted to the late responders in methylation apart from individual terms for early (*metabolism of vitamins and cofactors*) and middle (*immune system*), respectively.

A further analysis of the enriched pathways suggested the shared pathways to be largely related to cell homeostasis and activation including terms such as *cytokine signalling*, *innate immune response*, *inflammation,* and *response to stress*. Additionally, these shared pathways included several terms organised under categories cell cycle, metabolism of proteins, gene expression (transcription), and signal transduction (Figure 10). Although these categories were also represented among the pathways enriched uniquely for the transcriptome, the transcriptomic effects also included pathways suggesting acute effects such as *apoptosis* and *DNA damage response* (data reported in the Supplementary material of **Study III**).

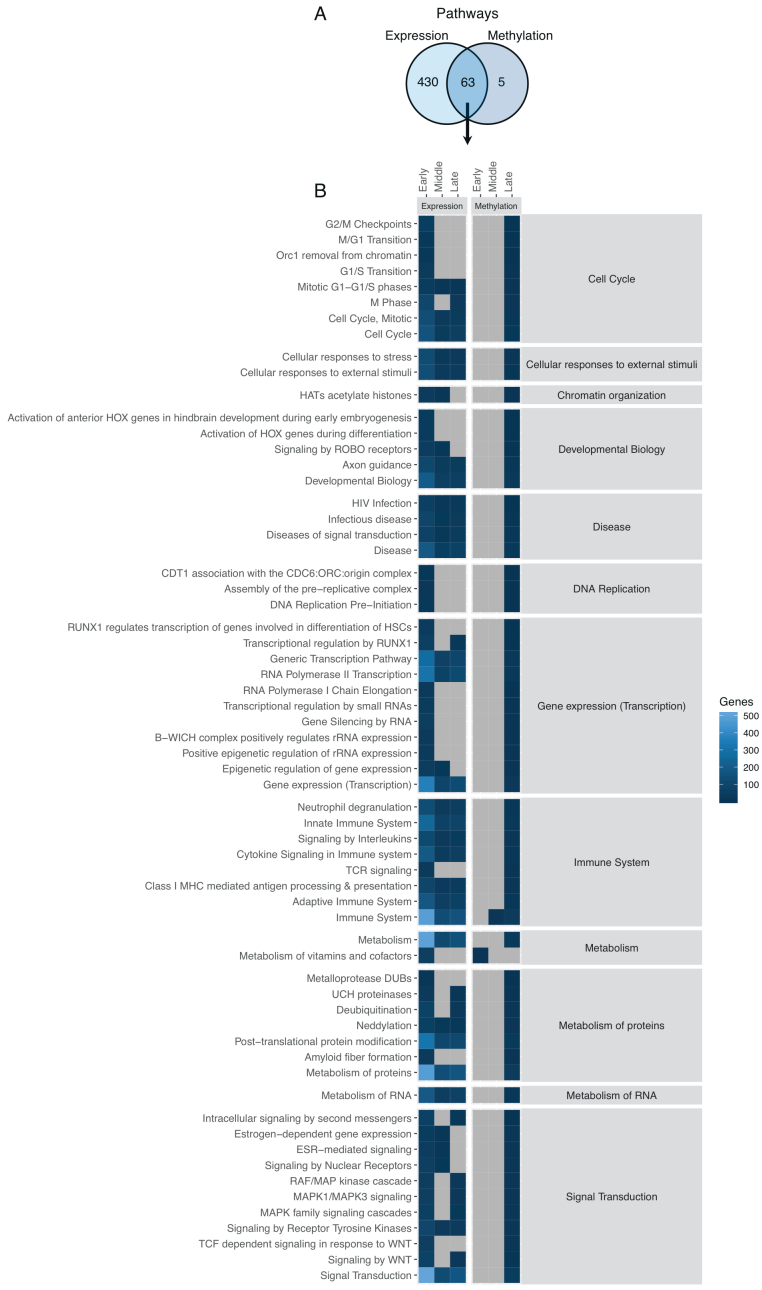

Figure 10. Comparison between the dMOA over gene expression and promoter methylation at the level of enriched pathways. A. Venn diagram depicting the overlap between the enriched pathways in the dMOA of transcription and promoter methylation. B. Enriched pathways shared by the transcription and promoter methylation. Figure originally published in **Study III**.

Finally, the results were evaluated in the context of pulmonary fibrosis (PF), one of the best characterised pathologies associated with MWCNT exposures. This investigation focused on assessing the genes and biological functions/pathways known to play a role in the pathogenesis of MWCNT-induced PF. The transcriptional dMOA revealed an overlap of 55/138 genes when compared to a previously established set of genes associated with the pathogenesis of PF in an *in vivo* model (Nikota et al., 2017). Some of the genes and relevant signalling pathways found altered in the dMOA are summarised in Figure 11.

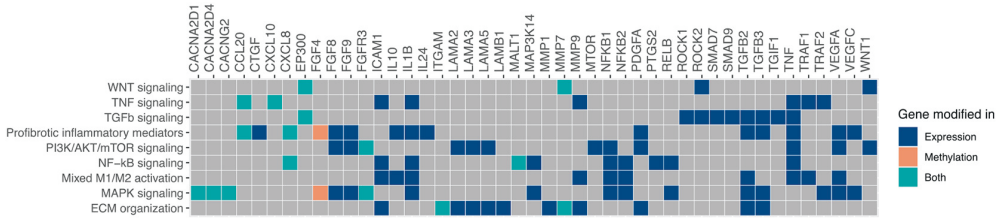

Figure 11. Selected pathways and genes relevant to the pathogenesis of MWCNT-induced pulmonary fibrosis. The heatmap represents the associations between selected genes of the dMOA characterised in the experiment and signalling pathways/biological functions. Dark blue squares indicate the gene to be part of the dMOA in expression, orange in promoter methylation and turquoise in both. Figure originally published in **Study III**.

5.2.3 Rigorous annotation improves the functional properties of AOPs

Functional properties of data were also investigated in **Study V**, where the systematic AOP-gene set annotations established in **Study IV** were evaluated in practice. In particular, the functional properties of the AOP-related annotation were investigated by assessing the potential to identify relevant adverse outcomes for a set of chemicals with known toxicity profiles using their associated molecular signatures. This approach showed that enrichment analysis against AOP-associated gene sets was able to highlight relevant adverse outcomes for all sex hormone receptor agonists (SHR) used in the study (Figure 12A). Similarly, the majority of the gene signatures associated with the carcinogens (50 out of 54) and known liver toxicants (seven out of eight) were able to highlight relevant AOPs for these chemicals. On the other hand, the approach did not work equally well for known thyroid disrupters, with thyroid-relevant adverse outcomes ranking at the top of the list for only three out of the seven chemicals included in the analysis (Figure 12A).

Furthermore, the annotations established in **Study IV** were used to investigate ways to group KEs. Such grouping could refine network applications of AOPs by addressing redundant nodes that may hamper the connectivity of the network, masking potential connections between currently disconnected KEs.

Initial grouping of all the KEs based on their gene set similarity revealed several reasons for KEs to group together. These reasons included 1) true duplication of the KEs; 2) the same event in characterised in distinct biological systems; 3) subsequent or connected KEs mapped to the same terms due to insufficient specificity; and 4) opposite regulation of the same biological event (e.g., increased vs. decreased signalling), where the last case is also due to the lack of specificity in the available gene sets. Given the large number of KEs and groups resulting from this practice, the topic was further investigated by focusing only on the AOPs related to PF, AO of high relevance in nanotoxicology (Bonner, 2010).

The collection included six PF related AOPs (*Aop:173, Aop:206, Aop:241, Aop319, Aop347, Aop:382*) with a total of 30 KEs mapped to them (Figure 12B). When modelling these AOPs as a network using the KEs as nodes and the KERs as edges, four distinct KEs were shared by different AOPs. Similarly, there were two independent nodes denoting the AOs (i.e., PF) (Figure 12C). However, the assessment of the KE similarity revealed six instances of potential duplication (Figure 12B). These groups were used to merge the redundant nodes to obtain the refined PF network as shown in Figure 12D. As a result of the merging, only a single node denoting the AO remained while seven of the KEs are now shared between two or more AOPs. Similarly, one of the AOPs (*Aop:206*) completely merged into the other AOPs.

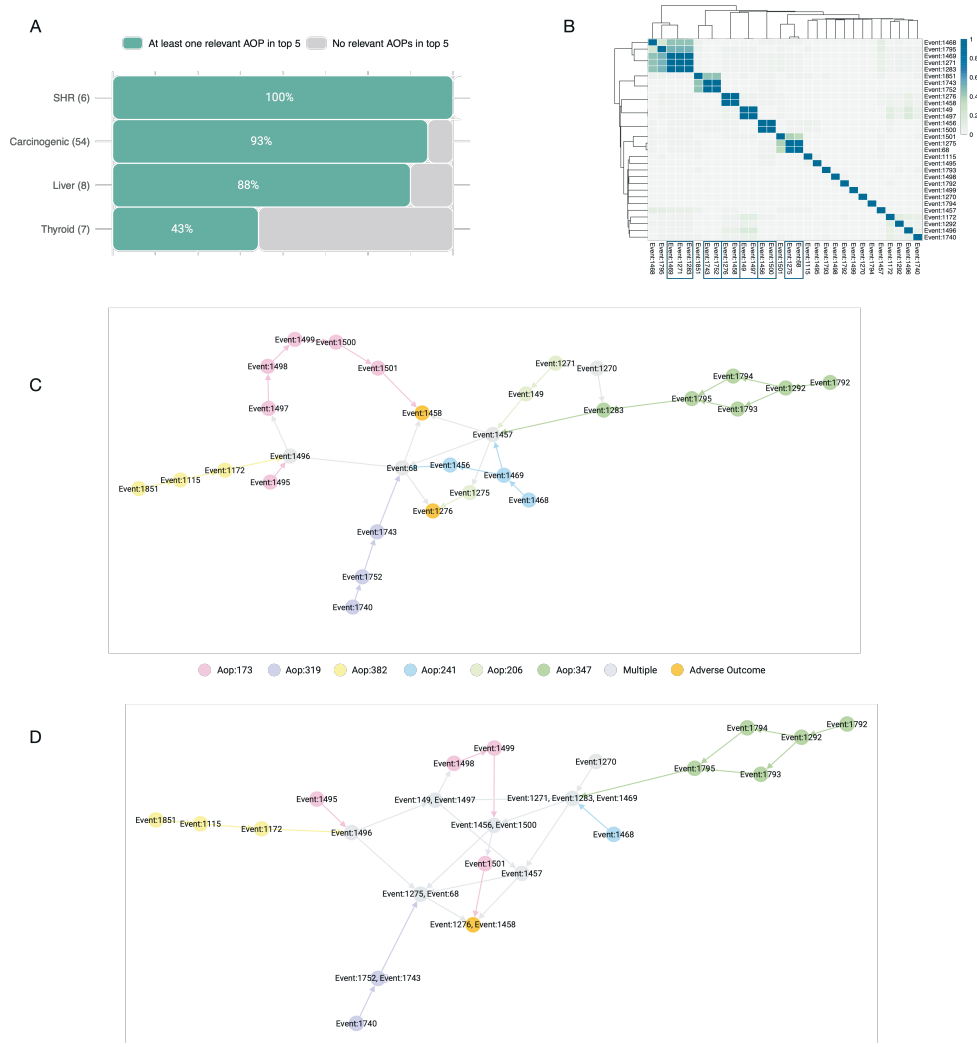

Figure 12. AOP annotations improve the functional properties of toxicogenomics data and AOPs. A. Bar chart indicating the proportion of chemicals showing relevant AOPs among the top enriched titles in the enrichment analysis against the AOP-associated gene sets. Number in brackets indicates the number of chemicals in the category. B. Heatmap representing the similarity of the PF KEs based on the Jaccard Index based similarity of their associated gene sets. Light grey represents low similarity scores, while increasing similarity is expressed with the colour changing through green to blue. C. Network presentation of the PF AOPs using their original KEs. Different colours represent the KEs of distinct AOPs and grey nodes denote KEs shared by multiple AOPs. Orange nodes correspond to AOs. D. Network presentation of the PF AOPs after grouping of the KEs. Figure adapted from **Study V**.

5.3 Translational properties of data

The final aim of this thesis was the investigation of translation properties of toxicogenomics data to define systematic strategies to translate toxicogenomic analyses into tangible information and specific NAMs for chemical safety assessment. The goal of this approach was to improve the interpretability of complex omics outputs even without advanced knowledge of toxicogenomics and computational biology while also answering to specific questions of toxicological importance.

Translatability of toxicogenomic data is enabled by the combination of the intrinsic and functional properties. Data that is intrinsically sound and appropriately annotated as well as presented in an adequate format is a prerequisite for functional data. The functional properties, on the other hand, further feed the translatability. This hypothesis was addressed in **Study V** where the intrinsic (AOP annotation) and functional (AOP-based analysis of toxicogenomic data) were used to define the concept of an AOP fingerprint as well as a systematic approach of selecting AOP-informed transcriptional biomarkers.

5.3.1 The AOP fingerprint shows high concordance between *in vitro* and *in vivo* exposures

The interpretation of the complex signatures captured by omics technologies remains one of the main challenges in toxicogenomics. Although methods such as functional enrichment using well established pathways and gene ontology terms serves as a common way to interpret transcriptomics data, these results require further interpretation to put them into the context of toxicologically relevant responses. This challenge was partly addressed through the AOP-based analysis of toxicogenomic data described in the Section 5.2.3. However, the concept was developed further into an approach referred to as the AOP fingerprint.

The AOP fingerprint was benchmarked using transcriptomic data from *in vitro* and *in vivo* exposures with a hazardous MWCNT, the Mitsui-7, known to induce PF upon pulmonary exposure (Porter et al., 2010). The *in vitro* signature comprised data from four distinct cell lines representative of the human lungs while the *in vivo* data was derived from mice lungs exposed to the same nanomaterial. The fingerprint was generated independently for both data sets.

As a result, 12 AOPs were found enriched *in vitro*, while 32 were enriched *in vivo*, with 10 AOPs being shared between the two sets. When ranking the results by the adjusted *p*-value from the most significant to the least, the top enriched AOP in both instances was the *Aop:173* titled "Substance interaction with lung resident cell membrane components leading to lung fibrosis". Similarly, the AOP at the second position (*Aop:171* – Chronic cytotoxicity of the serous membrane leading to pleural/peritoneal mesotheliomas in the rat) was shared by the two experiments. The remaining AOPs shared by the two sets included several AOPs describing to cancerous processes, PF, and respiratory distress.

AOPs unique to the *in vitro* data set (two AOPs) reflected specific processes, namely, disturbance of oxidative phosphorylation and IL-1 receptor related processes (Figure 13). The AOPs observed only *in vivo*, on the other hand, captured additional AOPs related to PF and various types of cancer as well as AOPs related to immune activation and gastric ulcer formation, for example.

Figure 13. AOP fingerprint of Mitsui-7 exposure *in vitro* and *in vivo*. The size of the dot corresponds the proportion of the significantly enriched KEs in each AOP (rows) and the colour represents the FDR-adjusted *p*-value in a negative logarithmic scale. The AOPs are sorted by the *p*-value *in vivo*. Figure originally published in **Study V**.

5.3.2 AOP-derived mechanistic *in vitro* biomarkers for pulmonary fibrosis

Biomarkers have emerged as a tool to monitor and predict toxicological effects of exposures. In the context of mechanistic toxicology, these effects can be KEs or other (molecular) events of interest. Although omics technologies have been used to identify molecular biomarkers for various pathological states and toxicological processes, they are often selected with little mechanistic context. The identification of biomarkers was addressed in **Study V** by defining characteristics for optimal transcriptional biomarkers based on the Bradford-Hill criteria for causation and applying them to identify a panel of biomarkers for PF.

The Bradford-Hill criteria were adapted to fit the purpose by considering the technology and methods available and suitable for chemical safety assessment. The original characteristics from (Hill, 1965) as well as their counterparts defined in **Study V** are listed in Table 4 together with a method of assessment and consideration for each characteristic. Some of the characteristics remained true to the original, some were adjusted, while two new characteristics were introduced: the method of assessment needed to be GLP-compliant (i.e., RT-qPCR for assessing changes in gene expression) and influence, which refers to the idea used in network science, where nodes that are more central are thought to be more influential as well.

Table 4. Characteristics for optimal biomarkers based on the Bradford Hill criteria. The original nine criteria have been modified and supplemented with additional considerations. Table has been originally published in **Study V**.

Bradford Hill	Biomarker characteristic	Method/Assessment
Consistency (reproducibility)	Reproducibility	Selection considers evidence from previous profibrotic exposures
Strength (effect size)	Amplitude	Significant alteration of the expression as compared to control
Experiment	Measurable	Transcriptional biomarkers measurable by qPCR; selected genes need to be expressed in the model
Biological gradient (dose-response relationship)	Dose-responsive	Benchmark-dose modelling to evaluate dose-response
Coherence	*In vitro* to *in vivo* extrapolation	Experimental evidence from *in vitro* and *in vivo*[1)]
Analogy	Predictive (of the outcome of interest)	Selection based on the KEs preceding the AO of interest
Specificity	Specificity	Gene ranking based on the specificity score
Plausibility	(Biological) plausibility	The AOP framework provides a plausible context; supporting evidence; selection of the organism
Temporality	Temporality	Transcriptional alteration follows the exposure; selection of the model organism[2)]
–	GLP-method	RT-qPCR
–	Influence	Centrality measures from human protein-protein interaction and gene regulatory networks

[1)]The biomarkers selected here are targeted for the development of non-animal assays for toxicological assessment. Hence the coherence to *in vivo* set ups is not evaluated experimentally. However, *in vivo* data was used for the selection of the markers to provide context of the systemic response. [2)]Temporality in the Bradford Hill criteria refers to a clear distinction of the exposure happening prior to the outcome. Here, we considered temporality by observing transcriptional changes post exposure as well as in the selection of the model organism. Macrophages have a crucial role in the initiation of the profibrotic response preceding the outcome, fibrosis.

The characteristics were considered in the prioritisation and selection procedure. The final selection comprised 25 genes out of the initial 2,075 genes associated with the KEs of all the PF AOPs (see Section 5.2.3.). These genes were validated by RT-qPCR, by evaluating the changes in their gene expression changes in an *in vitro*

macrophage model (PMA-differentiated THP-1 cell line) exposed to bleomycin, a profibrotic chemical.

The expression of 22 of the 25 genes was detected in the assay at one or more of the time points (6, 24 and 72 h) evaluated, while six genes were significantly deregulated (Figure 14). Finally, a monotonic dose response was one of the desired characteristics for the biomarkers, and this was fulfilled by five genes at one or more time points. These five included CXCL2 and CCL7 at 24 hours, IL8 (CXCL8) at 24 and 72 hours and MMP19 at 72 hours (Figure 14). Each of these genes was upregulated as compared to the controls (data available in the Supporting Information of **Study V**).

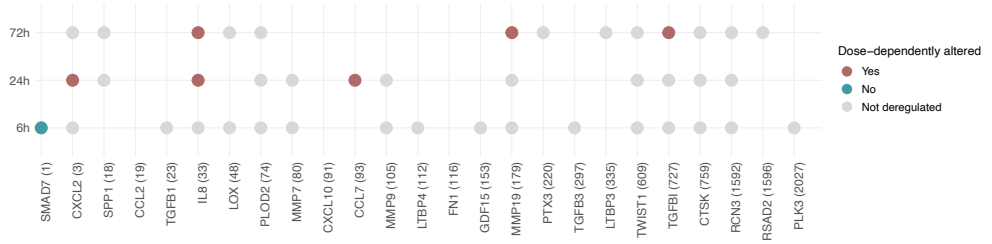

Figure 14. Dot plot depicting the results of the RT-qPCR validation of the biomarker candidates. Tested genes are visible on the x-axis with the number in the brackets corresponding to the final rank of the gene. Grey dots represent the genes whose expression was detected but not significantly altered between the exposed and control cells. Green dots reflect the genes that were significantly altered but not considered dose-dependent while the brown dots correspond to the final biomarker candidates that were both significantly altered and dose-dependent.

6 DISCUSSION

The overall aim of this thesis was to investigate the possibility to increase and ameliorate the application of toxicogenomics in chemical safety assessment. In accordance with the global movement towards alternatives to animal experimentation and more thorough, mechanistic assessment of chemical exposures, toxicogenomics holds the roadmap to transforming chemical safety assessment from an observational exercise to an integrated, descriptive field. The molecular mechanisms uncovered by toxicogenomics support early hazard identification, predictivity of models, and provide invaluable input for the implementation of the SSbD framework (Gomes et al., 2022; Liu et al., 2019).

However, full adoption of toxicogenomics into chemical safety assessment has been hampered by the lack of standardisation resulting in concerns of robustness and reproducibility, as well as the challenges in interpreting the complex output of omics technologies (Pain et al., 2020). While clear standards and guidelines regarding the generation, handling, and analysis of toxicogenomics data are still missing, efforts to support the robustness and reproducible analysis of toxicogenomic data have been the focus of numerous previous studies (Marwah, 2019; Serra et al., 2022) (add others!). These efforts have generally addressed individual aspects of toxicogenomics, focusing on experimental details, development of analytical pipelines or tools to analyse and support the interpretation of the resulting signatures (Di Lieto et al., 2023; Marwah et al., 2019; Scala et al., 2019).

In this dissertation, three distinct aspects of toxicogenomics data were investigated: its intrinsic characteristics, functional properties, and translational potential. Each of these constituents were investigated with the goal to support the systematic integration of toxicogenomics into chemical safety assessment through improved robustness, reproducibility, and ease of interpretation.

6.1 Intrinsic characteristics of toxicogenomics data

Intrinsic characteristics of data were defined as the FAIRness and overall availability, quality, and useability of the data. These characteristics arise from the details of the

experimental design combined with the decisions taken during data generation and analysis as well as the reporting of each of these steps and the resulting outcomes. However, considerations regarding data FAIRness are often limited to the metadata at the expense of data quality and its implications on the other aspects considered here as the intrinsic characteristics of data. These intrinsic characteristics are not unique to the metadata or the raw output of the experiments but extend over the analyses performed on these data and models built based on them. These aspects were the focus of **Studies I, II** and **IV**, of which **I** and **IV** were centred around data curation, providing a thorough look into the landscape of data in the field.

6.1.1 FAIRness is not an indication of data quality

Study I reported the curation of publicly available transcriptomic datasets from ENM exposures performed *in vivo* or *in vitro*, limiting the search to human, mouse, and rat data. Nanosafety data provided an interesting focal point for the study given the lack of unified data collections or reference data sets specific for ENMs (Grafström et al., 2022). Although several nanomaterial-specific databases have been established, they have mostly focused on storing physicochemical characteristics and exposure details, lacking harmonised, ready-to-use omics data (Jeliazkova et al., 2015; Krug, 2022; Yan et al., 2020). Such datasets are particularly important in the field of toxicogenomics, where the integration of multiple datasets is often needed to increase statistical power and broaden the applicability domain of the resulting models.

The curated collection of toxicogenomics datasets from **Study I** is the largest unified assembly of transcriptomics data measured upon ENM exposure to date. Although the integration of the data is still limited by the diverse experimental designs used in the individual studies, test systems, and gaps in material characteristics, the collection has enabled meaningful analysis, with a recent meta-analysis resulting in major discoveries concerning the first epigenetic-based One Health model of ENM response (del Giudice et al., 2023).

Although the curation and harmonisation of the data improved the FAIRness and usability of the existing datasets, the process also revealed several areas of concern. The quality assessment applied on the data resulted in the exclusion of one third of the originally identified datasets, with problems related to the experimental design being the most prominent reason (**Study I**). This implies that numerous toxicogenomics datasets featured in peer-reviewed publications exhibit significant

design flaws that could compromise the credibility of any findings derived from them.

The lack of replicates was the principal reason of failed quality assessment. Although the number of replicates used in an experiment depends on various aspects, such as the level of biological heterogeneity and anticipated amplitude of differences measured between experimental groups, three (biological) replicates are generally considered the absolute minimum to allow any calculation of statistical significance. As many as 26 datasets identified during the curation failed this minimum requirement. This problem is not unique to nanosafety, toxicogenomics, or any specific field employing omics technologies, but instead, it is part of a larger problem in modern science. Not having enough replicates hampers the robustness of the observation hindering reproducibility in science, a topic that has been heavily discussed in the recent years (Fanelli, 2018; Peng, 2015). In toxicogenomics, the cost of omics profiling is often accounted as the limiting factor, forcing compromises on the experimental design. However, proper experimental design could increase the validity and robustness of the data while also supporting data reuse, eventually decreasing the use of resources by reducing the need for new experiments.

While the number of replicates is often compromised due to the costs associated with omics technologies, several other experimental design flaws could be solved with little or no financial investments. In microarray experiments, the microarray slides and the dyes used to label samples often serve as a major source of batch effects (Kinaret et al., 2020b). Indeed, the lack of dye swapping was identified as the second most common design flaw among the identified datasets (**Study I**). This means that the experiment was implemented so that RNA extracted from distinct experimental groups were labelled with different dyes (lack of dye swapping, e.g., control samples with one and treated samples with another). This type of implementation prevents the identification of true biological differences between the sample groups as it is impossible to distinguish between the technical variation arising from the dyes and that accounted for the treatment of interest. Although this problem is unique to microarrays with a dual dye design, other systematic and often inevitable sources of batch effects work in a similar manner, necessitating careful consideration of the technical aspects of omics experiments to ensure the lack of correlation between technical and biological variables. This means that the potential sources of batch effects need to be identified early in the experimental design to pre-emptively control them through sample randomisation, for example. Sample randomisation can be assisted by dedicated computational solutions that evaluate correlations between technical and biological variables (e.g., Sinke et al., 2021).

Avoiding these correlations enables the mitigation of remaining biases during data processing using batch effect correction algorithms, such as the *ComBat* algorithm (Leek et al., 2012).

Recognising the importance of experimental design and batch effects is crucial for the generation of robust and intrinsically sound data. However, when it comes to data reuse, the details of the experimental design need to be justified and reported, an aspect that was found systematically lacking during the data curation (**Study I, II**). Although the source databases reinforce minimum reporting standards for omics experiments intended to support data FAIRness, several aspects of the data remained unreported. These technical details included potential sample handling dates, batches, operators, and slide numbers, to name a few. Hence, despite defining a set of minimum requirements for the data in **Study I**, systematic evaluation of biases in the data was often prevented by the lack of reporting on technical variables. Although algorithms, such as the *sva* algorithm, intended for surrogate variable analysis, can identify hidden sources of variation in high content data, they are not able to rescue a dataset where the source of bias is confounded with biological variables (Leek et al., 2012). Therefore, the validity, reproducibility, and reuse potential of published data can be diminished by the lack of transparency in reporting even when adhering to community-accepted minimum standards.

These observations suggest the need to update the data reporting standards. The importance of metadata cannot be emphasised enough. Instead of limiting the minimum requirements to primary variables, such as exposure doses and time points, complete documentation should be advocated. This documentation extends to experimental protocols and analytical details. These specifications would guide the reuse of the data while also determining its overall usability – whether the data is appropriate for the intended reuse purpose. To this end, distinction between technical FAIRness, such as that defined by the original FAIR data principles, and scientific FAIRness have been suggested (Papadiamantis et al., 2020; Saarimäki et al., 2022). While the original FAIR data principles serve as the guidelines on how to share data, scientific FAIRness can be reached through the definition of standard operating procedures (SOPs), complete metadata and clear data management practices (Papadiamantis et al., 2020).

Although these notions are important for all types of data, SOPs aimed at generating robust, high-quality data are crucial for increasing confidence towards toxicogenomics, where clear standards are still lacking. **Study I** highlights this shortcoming not only as the number of datasets deemed unfit for the collection, but also through the systematic lack of metadata and reporting. Finally, **Studies I** and **II**

underscored the importance of rigorously assessing data even if they adhere to all the FAIR principles. Acknowledging and addressing the aspects discussed here can serve as the stepping stone towards toxicogenomics data whose intrinsic properties enable robust and reproducible models, further increasing confidence towards toxicogenomics-based evidence in chemical safety assessment.

6.1.2 Robust annotation supports the application of AOP-based data in chemical safety assessment

Annotation is an important aspect of the intrinsic characteristics of data. Annotation is often used to categorise and label data for improved machine readability and machine learning applications, but it can also support the overall use, integration, and interoperability of data. Although the discussion on the FAIRness of AOPs falls beyond the scope of this dissertation (the topic is thoroughly discussed in recent publications such as (Mortensen et al., 2022; Wittwehr et al., 2023), the annotation effort described in **Study IV** also supports the FAIRness of AOPs. More importantly, however, it established the much-needed link between toxicogenomics and AOPs. The annotations enable contextualisation of toxicogenomics data and support its interpretation. Similarly, the gene-KE links unleash various analytical approaches that are further discussed in the following paragraphs.

AOP-Wiki supports various types of annotations, including the annotation of relevant gene ontology terms to KEs. Such annotations, however, are largely missing in the current database (https://aopwiki.org, *last visited in November 2023*). Furthermore, those present in the AOP-Wiki are typically rather generic, allowing categorisation of KEs but not intended to represent the biological events as corresponding gene sets. Previous attempts to annotate KEs to relevant pathways or gene ontology terms have been either focused on specific examples or remained at the level of theoretical associations limited to individual databases and not resulting in representative gene sets (Martens et al., 2022, 2018; Nymark et al., 2018).

In **Study IV**, the KEs of all AOPs relevant to human health were considered. This selection was based on the taxonomic applicability defined for most of the AOPs, selecting those applicable to humans or the most commonly used model species, such as rodents or primates. The annotation itself was based on an integrated approach combining NLP to prioritise relevant matches and manual curation to ensure appropriate context for them. Although the human-based assessment allows

for thorough quality checks and appropriate contextualisation of the associations, such manual processes are prone to potential interpretation errors and differences in views of priority and suitability of the matches. Expanding the annotations to various pathway and ontology databases provided flexibility and improved the probability of finding appropriate and accurate matches to the KEs. Similarly, including up to five annotations for each KE resulted in improved specificity and contextualisation when a single gene set would not result in a comprehensive match. This approach, however, relies on the robustness of the original gene sets.

Gene and protein annotation bias is a recognised challenge in biomedical research, likely affecting all the databases used in **Study IV** (Haynes et al., 2018). This refers to the fact that not all genes are equally-well characterised, and generally those already well annotated continue to be more readily researched (Haynes et al., 2018; Schnoes et al., 2013). It is also worth noting that the source databases (GO, KEGG, REACTOME, WikiPathways and HPO) have different strategies to curate gene annotations. To this end, similar terms in different databases may be associated with distinct sets of genes. Moreover, the KE-gene associations may change as the knowledge on the gene functions increase and the original gene sets are updated. Similarly, AOPs themselves are evolving and many of them are still under development. This is an important notion to consider when using the annotations, highlighting the need to refer to the original database and other resources along the way.

The KE-associated gene sets established through the curation are intended to provide a comprehensive link between the AOP framework and omics data in the light of current knowledge, supporting the analysis and interpretation of toxicogenomics data as well as the development of new AOPs. Thus far, the role of omics data in the application of AOPs in chemical safety assessment has been limited to the identification of potential MIEs and to provide supporting evidence for the pathways of toxicity (Brockmeier et al., 2017; Guan et al., 2022; Jin et al., 2022; Labib et al., 2016; Perkins et al., 2022). Hence, the systematic integration of these complementary concepts has been limited by the lack of connections between the molecules (e.g., genes and proteins) and KEs/AOPs. The methodological link established in **Study IV** paves the way for prioritisation strategies through the inference of potential adverse outcomes from individual exposures without the need to screen a panel of potential KEs with independent assays. Leveraging the network properties of AOPs, potential AOs can then be extrapolated from the data, and the testing of these outcomes can be prioritised (Knapen et al., 2018; Villeneuve et al., 2018). Although the annotations may enable the evaluation of multiple KEs from a

single omics experiment, the gene sets are not intended to replace the individual assays targeted for measuring specific KEs. This would require further refinement and validation of the gene sets to understand the gene expression patterns corresponding to the KEs as well as improved quantitative understanding.

One way to refine these initial gene sets and biological events captured by the KEs is through the biological context annotations also curated in **Study IV**. Although these annotations were already established in the AOP-Wiki with a fairly high coverage (data not shown), the existing annotations were refined, gap filled, and expanded to improve the accuracy and applicability domain of the KEs. For instance, the generalisability of certain KEs was improved by introducing a broader annotation that considers also biological plausibility. It has been previously noted that these types of annotations can serve as a guide for model development by defining in which biological compartments the AOP operates (Wittwehr et al., 2017). Hence, amending these annotations could enhance the accuracy of AOP-driven models and result in improved reuse of KEs. Here, the reuse of KEs refers to using the same KE in a newly developed AOP instead of establishing new KEs describing the same biological events. However, if the KE itself describes a process that could take place in various cell types or tissues, but the annotation is restricted to a specific biological compartment, the AOP developers are likely to create a new KE. This, in turn, results in disconnected nodes in the AOP network. On the other hand, including all possible biological systems for a KE allows their use as attributes for the AOP network, further enabling the filtering of the network based on these attributes if only a specific biological context is of interest. This way, refining the nodes (KEs) and removing redundant or duplicated KEs can lead to the discovery of hidden links between KEs as well as overall improved usability of the AOP network. This of course necessitates robust KERs that connect independent KEs in all possible cases. Together, these specifications define the intrinsic characteristics of AOP-related data, while their functional implications are further discussed in the Section 6.2.2.

6.2 Functional properties of toxicogenomics data

While the intrinsic characteristics of data build the foundation for subsequent analyses and models, the functional properties of data reflect how the data can be used and what type of information can be extracted from the use of the data. In one sense, the functional properties can be defined as the models and analyses built on

top of the foundation formed by the intrinsic characteristics. More importantly, these properties define the information that can be provided by the models.

The functional properties of toxicogenomics data are primed by the experimental set up. A dataset with multi-dose, multi-time point set up provides more modelling options than data with a single dose and time-point set up. Similarly, leveraging multi-omics data enables a more thorough characterisation of the molecular effects induces by chemical exposures. These data need to be further analysed and interpreted for meaningful biological insight. Hence, to build robust and informative models of chemical-biological interactions, the functional properties of toxicogenomics data were investigated in **Studies III** and **V**.

6.2.1 Multi-omics dMOA informs on the profibrotic potential of MWCNT exposure *in vitro*

One of the main challenges in the migration towards alternatives to animal experimentation is the prediction and assessment of long-term effects of chemical exposures. Although *in vitro* assays can sometimes be extended to long periods of time, they do not necessarily accurately reflect the events taking place *in vivo* (Alehashem et al., 2022; Donato et al., 2022). Mechanistic approaches can provide the tools to capture early events and enable models that have improved predictive value (Fortino et al., 2022). However, transcriptomics alone tends to offer a snapshot of the molecular mechanisms at the time of sampling, while the dynamics of the response are more difficult to model. To this end, **Study III** explored a novel way of analysing toxicogenomics data by combining transcriptomics and methylomics to obtain a multi-omics dMOA. This dMOA over the transcriptome and methylome could give insight into the kinetics of the molecular events while also explaining the regulatory mechanism of this response. Furthermore, the inclusion of DNA methylation could provide insight into the potential mechanisms of long-term adaptation, resulting in more informative *in vitro* models.

The time and dose integrated approach implemented in Serra et al. (2020) provides an alternative to more commonly employed BMD modelling (Serra et al., 2020b; Yang et al., 2007). While BMD modelling informs on the dose-responsive genes and enables the identification of PODs or other values relevant for chemical safety assessment, it gives little insight into the kinetics of the response due to the challenges in integrating results derived from independent time points. Nonetheless, dose-responsiveness is often thought as one of the indicators of causality and hence

used to distinguish between the core mechanism of the exposure from the complex omics output (Shimonovich et al., 2021). The TinderMIX approach retains this idea of the dose-responsiveness but solves the challenge of integrating multi-time-point data by simultaneously modelling the effects of the dose and time (Serra et al., 2020a). This further enables the identification of the time and dose combination that activates the monotonic change in the expression or methylation of the gene.

In **Study III**, the conventional analysis of differential expression and methylation upon Mitsui-7 MWCNT exposure suggested a prominent transcriptomic response, but DNA methylation was altered to a lesser extent. Similar observations have been made previously both *in vitro* (Öner et al., 2017; Scala et al., 2018; Sierra et al., 2017) and *in vivo* (Scala et al., 2021). This trend remained true when analysing the dMOA. While the dMOA highlighted an additional set of genes not deemed differentially expressed or methylated, it also underlined the stronger impact on transcription over methylation. Indeed, although DNA methylation is a dynamic process that can be affected by environmental exposures, the changes observed are generally small (Eze et al., 2020; Honkova et al., 2022; Messingschlager et al., 2023). Majority of evidence on the effects of environmental exposures comes from molecular epidemiology, where genome-wide changes in DNA methylation statuses have been analysed (Hannon et al., 2021; Hoang et al., 2021; C.-J. Xu et al., 2018). In these cases, causality is difficult to evaluate, and the focus is often on the changes in the overall methylation content instead of specific, individual regions. Here, on the other hand, the focus was on DNA methylation as a regulator of gene expression. Hence, changes in DNA methylation were investigated at specific regulatory regions, namely gene promoters, where it is thought to regulate gene expression through transcription factor (TF) binding. Although the classical model of TF binding on hypomethylated regions is not universal (Zhu et al., 2018), the role of DNA methylation changes in gene expression is generally well described. To this end, the overlap between the dMOA in transcription and promoter methylation was evaluated, showing that more than half of the genes whose promoter methylation was altered in a dynamic dose-dependent manner also had such changes in their expression. The kinetics of the responses across both molecular layers were further assessed by assigning the activation labels for the genes based on their PODs. Majority of these genes were those whose expression was altered early, but promoter methylation was affected late. This suggests a mechanism, where the change in gene expression is either sustained or supressed through DNA methylation as the exposure continues. Epigenetic changes in general are not among the first responders to acute exposures, but rather associated with repeated or long-term

exposures. This has been previously shown in the case of MWCNT exposures *in vivo,* where significant alterations in the DNA methylation levels in the lungs of mice exposed to MWCNTs were observed 7 days after the exposure, but not after 24 hours (Brown et al., 2016).

The distinct kinetics of the responses in the different molecular layers were also observed at the level of functional enrichment. Nearly all terms found enriched in the methylome were also significantly enriched in the transcriptome. In most cases, the term was enriched by the early genes in transcription (and either sustained or diminished by the middle and late genes) but only enriched by the late genes in methylation. While this is partly explained by the smaller set of genes in the early and middle groups as compared to the late group, it also supports the idea that DNA methylation plays a role in the regulation of core biological functions in sustained chemical exposures. This way, the system is adapting to the exposure by either altering its phenotype or priming its machinery to respond to a repeated exposure.

This phenomenon could also be observed in some key genes and pathways associated with MWCNT-induced PF. PF is one of the best characterised pathologies associated with MWCNT exposures (Labib et al., 2016; Nikota et al., 2017; Snyder-Talkington et al., 2016). While the development of PF is a complex process orchestrated by a collection of different cell types in the tissue, the role of macrophages in the initiation of the cascade has been described (Dong and Ma, 2018). They contribute to the production of reactive oxygen species (ROS) and initiate the inflammatory response driving fibrosis, provoking the profibrotic microenvironment in the tissue (Ogawa et al., 2021). Signalling pathways associated with NF-kB, TGF-beta and AKT/mTOR have been associated with fibrosis and were found affected in the analysis (He and Dai, 2015). The signature observed in the results is also suggestive of mixed status M1 (pro-inflammatory) and M2 (regulatory/healing) macrophage activation. This has been previously described in response to rigid MWCNTs both *in vivo* and *in vitro* and associated with MWCNT-induced fibrosis *in vivo* (Dong and Ma, 2018; Kinaret et al., 2020a; Meng et al., 2015). Here the dynamic dose-dependent alteration of genes encoding for proinflammatory factors such as IL-1β, CXCL-8, and TNF are suggestive of M1 activation, whereas the upregulation of profibrotic mediators CTGF, PDGFA, TGF-β2, and VEGF-A combined with the anti-inflammatory IL-10 suggest the activation of M2-macrophages. Moreover, a comparison of the affected genes with a previously established list of genes associated with MWCNT-induced PF *in vivo* resulted in an overlap of 55 genes (out of 138) (Nikota et al., 2017). Although the changes in promoter methylation did not affect all these genes, key factors, such as

CXCL-8 and MMP-7, were affected in both molecular layers. The early upregulation of the transcription was combined with late hypomethylation, suggesting persistent expression changes in these genes and similar patterns were observed in important biological processes involved in the pathogenesis of fibrosis, including cellular stress, calcium homeostasis and protein metabolism (Ryan 2014).

These observations provide insight into the molecular response of macrophages to MWCNT exposure, also highlighting the role of DNA methylation in changes associated with profibrotic potential of the exposure. Although the dysregulation of these biological processes has been previously characterised *via* conventional identification of DEGs (Kinaret et al., 2017b; Labib et al., 2016; Scala et al., 2018), the dynamic dose-dependent effect across both expression and methylation provides evidence towards more sustained regulation of these functions. Together, the identification of robust signals suggestive of the profibrotic potential of the exposure with evidence on sustained regulation of gene expression results support the use of *in vitro* systems in combination toxicogenomics to inform on potential long-term effects of chemical exposures from relatively short-term experiments. It further promotes the development of faster, cheaper, and more ethical testing strategies for chemical safety assessment while producing functional data and models. Finally, the exploring the epigenetic landscapes behind responses to chemical exposures could inform on mechanisms that may occur across multiple species, supporting the development of One Health models that no longer focus on individual exposures on specific biological systems (del Giudice et al., 2023; Saarimäki et al., 2023b).

6.2.2 Functional properties of AOPs are improved through data annotation

The interpretation of toxicogenomics-derived signatures can be facilitated by rigorously annotated AOPs. This was characterised in practice in **Study V** by evaluating whether the KE/AOP associated gene sets could capture relevant adverse outcomes from molecular signatures associated with chemicals of known toxicity profiles. These chemicals included hepatotoxic and carcinogenic agents, thyroid disrupters and known sex hormone receptor agonists (estrogen receptor, ER, and androgen receptor, AR). Sets of genes associated with these chemicals were retrieved from the CTD. It is worth noting that the CTD comprises chemical-gene associations from various sources and experimental set ups. Hence, these signatures do not necessarily reflect the MOA of a specific chemical in an optimal experimental

set up. Regardless, these gene sets can provide a general overview of the potential biological processes affected by these chemicals (Davis et al., 2019).

The results suggest that the enrichment approach successfully highlights relevant AOPs for each group of chemicals with SHR agonists performing best and thyroid disrupters the worst. These observations can be affected by multiple factors, including the number of chemicals in the final group, the number of relevant AOPs included in the dataset, and robustness of the gene associations in the CTD and in the curated data collection. Similarly, these factors can be affected by gene annotation bias (Haynes et al., 2018). Cancer-related processes have been widely researched and genes related to cancerous processes are often well annotated. Furthermore, although the chemicals are classified into one of these reference groups, other potential endpoints are not excluded. For instance, this was observed in the case of carcinogenic chemicals N-nitrosodiethanolamine and N-nitrosomorpholine. Neither of the gene signatures associated with these chemicals highlighted cancerous processes among the top enriched pathways. However, both pointed towards hepatotoxicity, with non-alcoholic steatohepatitis reported as a potential adverse outcome. Indeed, the AOP enrichment captured this effect in both cases (**Study V**). Similarly, some of the AOPs can be overlapping among the classification applied here. For example, *Aop:200* (ER activation leading to breast cancer) can be classified as both SHR related AOP and carcinogenesis related. Although this analysis supported the interpretation of gene signatures associated with chemical exposures, the signatures used here were not exposure specific and focused on the complete AOP, giving little insight into individual KEs. Hence, the concept was further developed into the AOP fingerprint, which is further discussed in Section 6.3.1.

While the enrichment analysis against AOP-associated gene sets can guide the interpretation of omics data, further enabling grouping and read-across approaches using mechanistic evidence, the KE annotations can also help the refinement of the AOPs themselves. This, in turn, improves the functional properties of AOPs and supports their use in combination with toxicogenomics.

One of the challenges observed in the AOP-Wiki was related to the duplication of KEs. Although creating a new KE can be valid in many instances, unnecessary redundancy can lead to challenges in the application of AOP-based knowledge. This is particularly true when modelling AOPs as a network and using such representation to identify hidden links or to perform read-across analysis (Arnesdotter et al., 2021; Clerbaux et al., 2022b; Knapen et al., 2018; Ravichandran et al., 2022). The

refinement of AOPs and the AOP network was assessed in **Studies IV** and **V** by evaluating the similarity of KEs based on the sets of genes annotated to them.

The analysis revealed that KEs cluster together due to multiple reasons (see Section 5.2.3) and the approach for defining the final groups depends on the intended application. For instance, considering all KEs mapped to the same set of genes as one is statistically justifiable for an enrichment analysis to avoid multiple testing against the same gene set, while retaining the granularity of the KEs is crucial for an AOP network. At the same time, removing truly duplicated nodes can be highly beneficial for network application (**Study V**).

The potential of this type of refinement was assessed by focusing on a subgraph formed by AOPs related to PF. The six AOPs available in the AOP-Wiki at the time of data retrieval characterise the multiple pathways leading to PF. Although the network was fully connected to begin with, the removal of the redundant nodes revealed new connections, reducing the number of nodes from 30 to 23. This further revealed the potential redundancy of an independent AOP as it was fully merged into the other AOPs upon refinement. The number of shared nodes increased from three to seven. Hence, refinement the AOP network simplifies the graph presentation while also enhancing the robustness of the KE relationships, depicted by the connections between the nodes. On a larger scale, this could lead to new paths across the network, linking disconnected parts to those more thoroughly linked, a finding whose significance can be postulated in the context of the whole AOP network (Villeneuve et al., 2018). Similarly, as duplicated KEs are removed, the true influence of each node can be evaluated more robustly through network analytics. This example highlights the effect of KE redundancy and the potential of data-driven grouping of KEs. As the AOP-Wiki keeps growing, the significance of data-driven approaches increases. Although manual assessment is feasible at the level of individual AOPs and events of interest, doing it AOP-Wiki wide is a massive undertaking. Hence, encouraging AOP developers to consider the potential of reusing KEs and carefully annotating the data would support future applications of AOPs.

Further refinement of the AOP network could be achieved through the biological system and taxonomic annotations. The biological systems (**Study IV**) and taxonomical annotations could be used to refine the network by only depicting the KEs of a specific organ system or reveal KEs shared by various organisms to find points of convergence or diverge. This has also been pointed out by Wittwehr et al. (2017) who note that "independent models may be needed to translate well-conserved effects of chemical perturbation on thyroid hormone concentrations to

divergent AOs across species" (Wittwehr et al., 2017). Increasing the understanding of the similarities and differences between AOPs in various species not only supports the development of more appropriate models for the assessment of human health risks and environmental risks, but also serves as a step towards holistic understanding of chemical hazard and the One Health perspective (Saarimäki et al., 2023b).

6.3 Translational potential of toxicogenomics data

Finally, the translational potential of toxicogenomics data is influenced by both the intrinsic characteristics and functional properties of the data and further supported by robust ways to translate the information into human-readable format and concrete assays that inform chemical safety assessment. Understanding the translational potential of toxicogenomics data supports the integration of toxicogenomics-based evidence into chemical safety assessment by improving the interpretability of the complex outputs while also resulting in approaches that enable more targeted data generation through the implementation of mechanistic NAMs.

6.3.1 The AOP fingerprint supports the mechanistic interpretation of toxicogenomics-based evidence

The interpretation of toxicogenomics data remains a major challenge. While various approaches to functional enrichment have facilitated the translation of the complex signatures into tangible biological pathways, their application to chemical safety assessment is not straightforward. Alteration of specific molecular pathways or biological processes informs on the subcellular mechanisms, but these need to be further placed in the context of toxicologically relevant pathways eventually leading to adverse outcomes. This laborious process requires extensive knowledge and manual literature searches but can often lead to various interpretations. Similarly, the increasing adoption of *in vitro* models necessitates the extrapolation of these results into *in vivo* relevant scenarios. The AOP framework can support this endeavour through the specific KEs that can be monitored via NAMs (Van der Stel et al., 2021). This way, the causality expressed by the AOP contextualises the evidence derived through alternatives to animal experimentation, improving the robustness of such evidence.

In this work, the KE-gene set annotations established in **Study IV** were developed into the AOP fingerprint. The fingerprint was benchmarked using *in vitro* and *in vivo* data from a comparable exposure, allowing the comparison of the signatures. Both data sets highlighted the *Aop:173* titled "Substance interaction with the lung resident cell membrane components leading to lung fibrosis" as the most significant AOP. While the multi-dose, multi-time point *in vivo* data used here showed histological evidence of this in the original publication after one week (Porter et al., 2010), the *in vitro* data presents a much more simplified experimental set up with the distinct cell types exposed to a single sublethal dose for 48 h. Regardless of the simplicity of the experiment, the data captures the profibrotic potential of the exposure in the form of multiple fibrosis related AOPs.

The second most significant AOP (*Aop:171*) shared by the two sets delineates the development of pleural/peritoneal mesotheliomas due to prolonged cytotoxicity in rats. *Aop:171*, much like the other AOPs included in this study, is in the process of refinement and currently lacks data on potential stressors. However, mesothelioma is a recognised AO associated with asbestos and evidence of MWCNT-induced mesothelioma has emerged (Mossman et al., 1990; Numano et al., 2019; Suzui et al., 2016).

Many of the other shared AOPs are also related to various cancerous processes. This suggests that the transcriptomic signatures both *in vivo* and *in vitro* capture biological processes suggestive of the carcinogenic potential of Mitsui-7. Indeed, Mitsui-7 classified as a potential carcinogen by the IARC (Grosse et al., 2014), and its carcinogenic potential is suggested to be related to the inflammatory reactions induced by pulmonary exposure (Rahman et al., 2017a). The inflammatory processes are also captured by the AOPs in the AOP fingerprint albeit more specifically in the *in vitro* data. Such specific signals might be easily masked in the *in vivo* system, where a large array of cell types is affected and screened simultaneously. The *in vitro* data further highlighted effects such as cytotoxicity, frustrated phagocytosis, and oxidative stress, all of which have been reported as consequences of this type of exposure and contribute to the pathogenic nature of Mitsui-7 (Kinaret et al., 2017a; Rahman et al., 2017a; Rydman et al., 2015). On the contrary, the *in vivo* fingerprint captured various AOPs suggestive of effects outside the respiratory system. Although the effects of pulmonary exposure to MWCNT are not necessarily limited to the immediate exposure site, these results could also reflect different effects of similar transcriptomic signatures in diverse biological systems. For instance, the AOPs related to gastric ulcer formation could suggest similar mechanisms of surfactant disturbance in distinct exposure sites.

The results of the fingerprint are likely affected by the differences between the two data sets. Firstly, the number of DEGs was notably different between the two data sets (863 *in vitro* and 3540 *in vivo*, respectively). Similarly, the experimental set ups were not comparable with each other. Nonetheless, the example here suggests that even a simple exposure set up *in vitro* can accurately capture AOs associated with long-term exposures *in vivo*. Furthermore, the convergence of the fingerprints supports the use of published data in the development of NAMs. Based on the histopathological evaluations associated with the *in vivo* data, the transcriptomics should capture the full PF AOP. Indeed, all KEs except the MIE in the *Aop:173* were enriched. The *in vitro* data, on the other hand, highlighted the same AOP as the most significant despite only enriching only a half of its KEs. Moreover, the high coverage over the relevant KEs provides evidence of the accuracy of the KE-gene set annotations, supporting the use of toxicogenomic evidence for the development of AOPs and for the evaluation of potential AOs of chemical exposures. Altogether, the AOP fingerprint shows that the analysis of toxicogenomic data against robustly annotated AOP framework supports a high degree of *in vitro* to *in vivo* extrapolation and further encouraging the inclusion of toxicogenomics-based evidence for chemical safety assessment.

6.3.2 The AOP framework provides mechanistic context for transcriptional biomarkers

In **Study V**, the Bradford Hill criteria for causality were modified into characteristics of optimal biomarkers. While the definition of a biomarker is broad and diverse, it can be generalised into a measurable indicator of a biological state or processes (Strimbu and Tavel, 2010). Typically, this means that the biomarker needs to influence or predict the incidence of an outcome or disease (Ipcs, 2001). Biomarkers can hence be clinical signs such as blood pressure measurements or molecular effects, like changes in gene expression. Although biomarkers can be categorised in various ways, including those defined as diagnostic, prognostic, and predictive, the most relevant categorisation for this dissertation is it distinction between descriptive or mechanistic (Califf, 2018). Descriptive biomarkers are a by-product of the disease state while mechanistic biomarkers inform on the pathogenesis of the disease (Robinson et al., 2013). Mechanistic biomarkers can monitor the pathogenesis or biological process before a disease state is achieved, resulting in actionable biomarkers. This also means that the biomarkers can be used to inform on potential

risks or effects pre-emptively or early in the process (Antoine et al., 2013). The need for mechanistic, actionable biomarkers has been recognised across various fields, including diagnostics, drug development and pharmacology, as well as toxicology (Carr et al., 2017; Davis et al., 2020; Fortino et al., 2022; Narayan et al., 2020).

In the case of the AOP-derived biomarkers sought after in **Study V**, biomarkers are genes that could inform on the mechanistic cascade of events leading PF and hence providing insight into the profibrotic potential of chemicals. The criteria were originally conceived to evaluate hypothesised causal relationships in epidemiology and have since been applied in the study of various exposures and disease outcomes (Breckenridge et al., 2016; Elcombe et al., 2014; Fedak et al., 2015; Mente et al., 2009; Tischer et al., 2011). While the nine characteristics are not intended as a checklist for causality, they serve as flexible guidelines to support epidemiologic investigations. Although the molecular mechanisms behind disease and exposure associated outcomes were largely uncovered in Hill's years, the characteristics have the potential to go past the "black box" investigation typical for classical epidemiology and toxicology, allowing data integration and mechanistic reasoning (Fedak et al., 2015).

In this work, the criteria were placed in the context of mechanistic toxicology. The nine characteristics were modified and supplemented with additional two: GLP-approved method and so-called influence. The former was set in place to enable the screening of the suggested biomarkers using robust and reproducible methods already employed in chemical safety assessment. In the case of transcriptional biomarkers, this method is hence RT-qPCR. Influence, on the other hand, was based on the social life of genes: some genes (or gene products) are more influential than others. They may serve as master regulators or as the link between important biological processes, for example. To this end, network analytics over gene or protein networks can be applied. The influence of a node (here gene/gene product) can be assessed through centrality measures often based on the degree, betweenness and closeness, among other metrics (Zhao et al., 2022). This idea is widely accepted and so-called hub genes are often suggested as potential biomarkers (Li et al., 2017; Zhao et al., 2022; Zhou et al., 2019). Here, influence, as defined by this concept, was used as one of the prioritisation metrics while it was supplemented by considerations of context, specificity, and experimental evidence.

Considering the aim of identifying mechanistic biomarkers that could inform on the chain of events leading to an AO of interest (PF), the AOP annotations from **Study IV** served as the context. This way, the genes associated with PF AOPs were used as the starting point and ranked according to the protocol and evaluated

experimentally. The full set of considerations is detailed in Table 4 (*cf.* Methods) and the initial ranking was based on the influence and specificity to enable flexible consideration of experimental data depending on its availability. Here, experimental evidence was used to support manual selection over the initial rank. The final selection of candidate biomarkers covered various rank positions to ensure the coverage over multiple PF KEs as well as the biological feasibility (i.e., selecting candidate genes that are likely to be expressed in macrophages).

The final set of genes that fulfilled the desired characteristics, including magnitude (significant alteration) and dose-dependent effect (BMD modelling), comprised CXCL2 and CCL7 at 24 h, IL8 (CXCL8) at 24 and 72 h, and MMP19 and TGFBI at 72 h. All but TGFBI were among the top 10% of the original rank, suggesting that the initial rank could support the selection of genes even in the absence of experimental data. The rank itself is influenced by the robustness of the data, both at the level of the AOP annotations and the PPI/regulatory network data used to evaluate the influence.

Many of these suggested biomarkers are known as chemokines that mediate immune responses. IL8 and CXCL2 are best characterised as neutrophil attractants, while CCL7 targets a wide variety of leukocytes (Cheng et al., 2014; De Filippo et al., 2013; Hammond et al., 1995). This indicates that the activation of the macrophages was captured at the level of gene expression. Prolonged inflammation together with persistent M2 macrophage activation has been linked to the pathogenesis of PF (Braga et al., 2015). Such polarisation has been previously associated with bleomycin. The anti-inflammatory M2 macrophage phenotype is thought to drive the development of PF through their ability to promote myofibroblast differentiation (Hou et al., 2018; H. Wang et al., 2023). Although important in the development of PF, inflammatory markers alone do not necessarily inform on the profibrotic potential of the exposure. However, the additional effect observed in the expression of MMP19 and TGFBI increase the specificity of this panel as PF biomarkers. MMP19 has been previously characterised as a key regulator of PF in both mice and humans, although previous efforts have mainly investigated it in the context of epithelial and endothelial cells (Yu et al., 2012; Zhao et al., 2023). It belongs to the family of metalloproteinases involved in the remodelling of the extracellular matrix (ECM). Similarly, the protein encoded by TGFBI is involved in the ECM. It has been shown to bind type I collagen leading to thicker fibres and further promoting M2-type macrophage polarisation (Bachy et al., 2022). The upregulation of TGFBI and SMAD7 (albeit not deemed dose-dependent and hence not part of the final panel) also indicate the activation of TGF-beta signalling. TGF-

beta is considered as one of the main markers of PF. However, neither of the TGFB genes (TGFB1 and TGFB3) tested in our experimental set up revealed significant changes in their expression. Indeed, TGF-beta presents a more attractive candidate for protein-based biomarker assessment, as it is activated from its inactive form post-translationally, meaning its activation may not be reflected in the transcription of the gene encoding the protein (Annes et al., 2003).

The activation patterns of these genes capture the cascade of events recognised in the development of PF. The inflammatory signal observed especially at 24 hours of exposure is followed by the activation of genes involved in ECM remodelling and signals associated with myofibroblast differentiation at 72 hours contributing to the development of the profibrotic microenvironment and responses of the other cells of the tissue (Braga et al., 2015). Although macrophages alone cannot capture all KEs of the PF AOP, the suggested biomarkers here represent the key steps in macrophage involvement in PF, supported by the context provided by the AOPs. While biomarkers are often identified based on a phenotype that has already been reached, the mechanistic reasoning offered by the AOP framework supports the identification of early events and biomarkers that would support predictive evaluation. This example highlights how the improved intrinsic and functional data characteristics enable the translational potential of both toxicogenomics and AOP data, resulting in mechanistic NAMs that could support chemical safety assessment.

7 CONCLUSIONS

Toxicogenomics has emerged as a powerful approach to uncover the molecular mechanisms behind the effects of chemical exposures. Although the potential of this mechanistic insight is widely recognised and has resulted in vast amounts of published data, toxicogenomics-based evidence struggles to be fully adopted into chemical safety assessment (Liu et al., 2019; Pain et al., 2020). The investigation in this dissertation highlighted the potential of data curation in understanding the current state and progress in the field, enabling a breakdown of the data and the potential challenges in its intrinsic characteristics that hinder the application of omics-based evidence in chemical safety assessment. Acknowledging and tackling the challenges recognised in each step of data generation and its reporting form the foundation of progress. This can be achieved through systematic approaches to data curation, FAIRification and the implementation of SOPs that support the generation of robust data whose intrinsic characteristics have been considered in the early steps of the experimental design. Once the generated data is robust, high quality and fit for purpose, its reporting forms the second step towards intrinsically sound data. This not only improves reproducibility in the field but also supports data reuse and other aspects of FAIR data, serving as a step towards more sustainable research. Finally, the intrinsic characteristics of data should not be limited to raw outputs of experiments but need to extend over the models built based on this evidence, underlining the importance of complete reporting throughout experiments and analyses.

The efforts undertaken in the context of this dissertation further highlight the value of data curation. This laborious task can serve as a means for a systematic review of both the intrinsic and functional characteristics of data and the current state of the field, resulting in better resources that enable novel discoveries (del Giudice et al., 2023). In a field like toxicogenomics, where the generation of unified large-scale reference datasets is limited by resources, these efforts are crucial and should be recognised as a valuable contribution. The review through curation underscores the heterogeneity in the produced resources, revealing the lack of standardisation in the field. This further emphasises the pressing need for robust standards for data generation and reporting.

Although the intrinsic characteristics of data can be improved through rigorous curation (**Studies I, II** and **IV**), these aspects should be considered prior to data generation. The limitations set in place by the initial experimental design or data entry have far-reaching consequences. This mirrors the idea of retrospective vs. pre-emptive characterisation between epidemiology and toxicology. While the insight provided by retrospective investigation is priceless, we cannot solely rely on retrospective evidence. Instead, we need to learn from the evidence in front of us and implement strategies to work pre-emptively and establish links between the vast resources available. Hence, the challenges uncovered in this dissertation should serve as a foundation towards the next generation of data that is intrinsically robust and whose functional properties enable meaningful discoveries.

These considerations are highly relevant also for the intrinsic characteristics of AOPs. The AOP framework rapidly gained importance in chemical safety assessment, establishing itself as one of the cornerstones of modern toxicology. AOPs serve as a natural anchor of various types of mechanistic evidence to chemical safety assessment, now also to toxicogenomics-based evidence. While the AOP-gene associations established in the context of this dissertation rely on the robustness of the data used and the semantic associations between concepts, the examples highlighted in the context of the functional properties and translational potential of data support the validity of these associations. The scientific community has recognised the potential of integrating these complementary frameworks (Labib et al., 2016; Nymark et al., 2018; Perkins et al., 2022). **Study IV** has now made this resource publicly available, hopefully inspiring various future endeavours.

The implications of the intrinsic characteristics of toxicogenomics data extend to its functional properties and translational potential (**Studies III** and **V**). Toxicogenomic investigation has primarily focused on the characterisation of transcriptomic alterations and the depiction of the MOA of chemical exposures (Kinaret et al., 2021; Poulsen et al., 2015; Rahman et al., 2017b). However, the generation of more informative models that thoroughly disentangle the interactions between chemicals and biological systems calls for experimental set ups and analytical approaches that improve the functional properties of data. Implementing models that explain the mechanisms beyond the gene expression could reveal molecular layers that expand the applicability domain of the model or enhance the biological insight provided by the model, supporting the use of non-animal approaches (del Giudice et al., 2023; Saarimäki et al., 2023b). The integration of the time and dose effect with insights into DNA methylation unveiled some of the mechanisms contributing to the profibrotic nature of the MWCNT exposure (**Study**

III), suggesting the value of such methods when developing *in vitro* models for evaluating potential long-term effects of various exposures. While an experiment should be designed to answer a specific research question, the limits and possibilities set by the design to the future reuse of the data should be acknowledged to support the efforts towards more sustainable research. The information content provided by the added complexity further necessitates analytical approaches that benefit from such experimental set ups.

Studies IV and **V** showed how the systematic link established through annotation improved the functional properties of both toxicogenomics data and AOPs, as well as the translational potential of toxicogenomics data. One of the most prominent challenges in the application of toxicogenomics data for chemical safety assessment is the interpretation of its complex output. The AOP framework serves to contextualise this evidence into toxicologically relevant events. Although the associations implemented at this stage are qualitative at best, the investigations in **Study V** shows the potential of this association. The AOP fingerprint summarises the omics-based signature into tangible pathways that can be further explored through the enriched KEs, expediting future quantitative approaches. Likewise, the mechanistic context granted by the AOPs for the identification of potential biomarkers for the evaluation of chemical hazard expands the avenues for the development of mechanistic NAMs. The framework can be further developed towards comprehensive panels of sentinel molecular entities that empower targeted data generation with reduced costs and higher throughput. With toxicogenomics anchored into the AOP framework, the future holds the promise of a more comprehensive and mechanistic understanding of chemical hazards, paving the way for the development NAMs that prioritise the safety of both humans and the environment, ushering in a new era of chemical safety assessment.

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PUBLICATION I

Manually curated transcriptomics data collection for toxicogenomic assessment of engineered nanomaterials

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Manually curated transcriptomics data collection for toxicogenomic assessment of engineered nanomaterials

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Toxicogenomics (TGx) approaches are increasingly applied to gain insight into the possible toxicity mechanisms of engineered nanomaterials (ENMs). Omics data can be valuable to elucidate the mechanism of action of chemicals and to develop predictive models in toxicology. While vast amounts of transcriptomics data from ENM exposures have already been accumulated, a unified, easily accessible and reusable collection of transcriptomics data for ENMs is currently lacking. In an attempt to improve the FAIRness of already existing transcriptomics data for ENMs, we curated a collection of homogenized transcriptomics data from human, mouse and rat ENM exposures *in vitro* and *in vivo* including the physicochemical characteristics of the ENMs used in each study.

Background & Summary

Engineered nanomaterials (ENMs) are an emerging class of chemicals with great technological and societal impact. Their unique physicochemical properties have already inspired multitudes of applications, ranging from medicine to industry and consumer products. While these unique properties make ENMs attractive for endless applications, they can also be responsible for potentially harmful effects on human health and the environment. ENMs can be synthesized in various sizes, shapes and chemistries with the smallest differences in the composition leading to novel properties and effects that need to be considered. Rigorous risk assessment is needed to ensure the safety of ENMs. Toxicogenomics (TGx) has emerged as a complementary approach to traditional toxicology with the potential to facilitate faster and cheaper hazard assessment of ENMs[1,2]. The large-scale profiling of exposure-induced molecular alterations sets the stage for mechanistic toxicology and expedites the development of predictive models. Furthermore, the application of TGx data to nanosafety can provide novel possibilities of grouping and classifying ENMs based on the similarity of molecular alterations in biological systems and furthermore can help to derive biomarkers to identify nano-specific signatures.

Transcriptomics technologies are the frontline of TGx. Vast amounts of transcriptomics data for multiple ENMs have already been generated offering a valuable resource for future studies and applications. However, the data are scattered across public repositories, and their FAIRness is currently hampered by their heterogeneous nature and lack of standardization in the preprocessing and analysis. The FAIR principles for scientific data were defined in 2016 and have since been the guide for more Findable, Accessible, Interoperable, and Reusable data[3]. The FAIRness of ENM-relevant databases, including ArrayExpress, the Gene Expression Omnibus (GEO), eNanoMapper and NanoCommons have recently been evaluated, and while the six datasets extracted from these met the majority of the criteria defined by the FAIR maturity indicators, areas identified for improvement included the use of standard schema for metadata and the presence of specific attributes in registries of repositories that would increase the FAIRness of datasets[4]. In order to unleash the full potential of already existing transcriptomics data

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Fig. 1 The workflow applied to compile the data collection. Solid-lined boxes represent the steps applied while the output is marked with a dashed line.

on ENM exposures, which are lacking the metadata related to the exposure conditions and ENM characteristics, we created a unified collection of 101 manually curated and preprocessed data sets, covering a range of ENMs, organisms, and exposure setups, using the approach represented in Fig. 1.

The overarching aim of this study was to manually curate a comprehensive collection of transcriptomics data in the field of nanosafety, thereby increasing the degree of FAIRness of the original data sets. In particular, our collection is characterized by a higher degree of FAIRness as compared to the individual original data sets composing it.

Methods

Data set identification and collection. The first step in compiling the collection was to identify relevant data sets across public repositories. The search was limited to human, mouse, and rat data. We queried the Gene Expression Omnibus (GEO) and ArrayExpress databases with the following search terms: "engineered nanomaterial", "nanomaterial" and "nanoparticle". The initial collection yielded 124 unique entries, which went through manual assessment. Raw, non-normalized data for each microarray-based entry was downloaded from the series entry page, while for RNA-Seq data sets raw sequencing data in .fastq format were retrieved from the European Nucleotide Archive (ENA) (https://www.ebi.ac.uk/ena/browser/home).

Metadata curation. Next, supporting information (metadata) for each entry in the initial collection was downloaded and manually curated on R (version 3.5.2). Metadata gives context to the data by mapping each sample to biological variables, such as dose and time point, as well as technical variables crucial for the preprocessing of the data.

Metadata were obtained from the sample records of GEO entries by using the function *getGEO* from the R package GEOquery[5]. For data sets available only on ArrayExpress, the sample information for each entry was downloaded. These data were then manually curated to produce a homogenized file for each data set consisting of the following variables: *GSE* (a unique identifier for each data set), *GSM* (sample id), *treatment* (exposure; *i.e.* ENM or control), *group* (experimental group; combination of a unique exposure, dose, and time point), *organism, biological system, dose, dose unit, time point, time point unit, slide, array, dye* and platform. Although some of these variables are not relevant for RNA-Seq data, all the columns were included for all the data to ensure convenient data usability. The nomenclature was unified to an extent that could be reached based on the information provided in the original metadata. Each sample was then mapped to its corresponding raw data file (column *filenames*) or annotated later to the fastq-files based on the sample names (GSM). If one or more predefined technical variables were missing, the column was left empty (NA). However, if biological variables were missing or ambiguous, the data set was discarded. Lastly, for entries containing human primary cells, the donor was further included in the metadata as an additional column *donor*.

ENM physicochemical characteristics curation. The majority of the datasets were associated with a published article describing the study and including some details of the materials used and their physico-chemical characteristics. In some cases, the information provided was the nominal size information from the ENM manufacturer, while others provided more detailed characterization of the ENM in the exposure medium. Newer studies tended to provide more detailed characterization information than older ones, as the community knowledge regarding minimum characterization needs and properties influencing ENM toxicity increased[6,7]. Several of the studies utilized ENMs already used in previous studies and referred to the characterization provided in those earlier studies, in which case the information was manually extracted from the earlier papers. The curated information for the ENMs includes information on the supplier (including batch and lot information where available), the purity / impurities, the nominal size and surface area, as well as characterization data such as the core particle size (shape) as determined by Transmission Electron Microscopy (TEM) size, the hydrodynamic size and zeta potential (surface charge) in water and/or the exposure medium determined by Dynamic Light Scattering (DLS), information on the presence of endotoxin contamination (where provided) and a link to the commercial providers material specification sheet where relevant. As many of the studies utilized several different ENMs, or several variants (e.g. sizes, capping agents, polymeric coatings etc.) each individual ENM within each study is described in a separate row of the ENM characteristics datasheet.

Manual quality assessment. The quality of transcriptomics data is highly dependent on the experimental design[2]. Low number of replicates results in weak statistics, while transcriptomics technologies themselves are often prone to technical bias. In order to ensure the quality and usability of each individual data set, evaluation was carried out based on the availability of raw data and supporting information as well as technical aspects of the

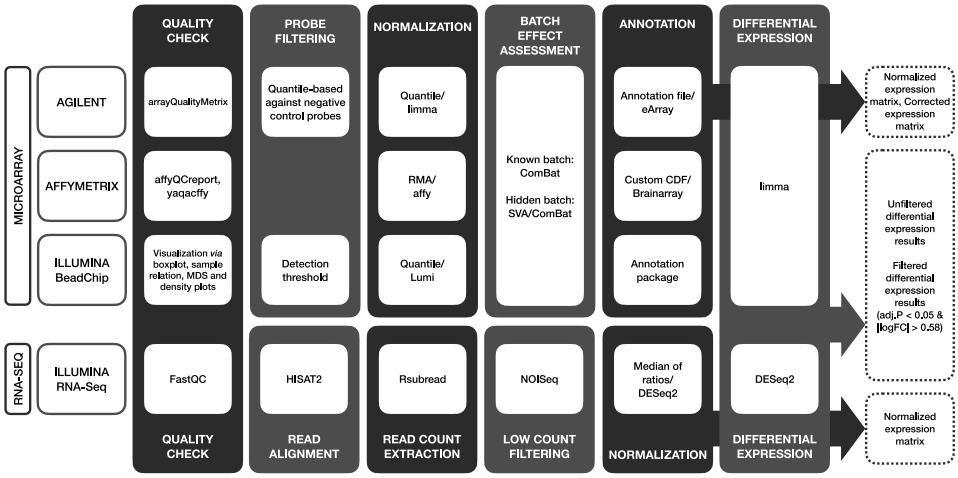

Fig. 2 Preprocessing workflow applied to Agilent, Affymetrix, and Illumina microarrays and Illumina RNA-sequencing. Boxes with a blue background represent preprocessing steps and methods applied for each platform while boxes outlined with a dashed line represent the output obtained for each data set. The lack of a white box indicates that the step was not applied for the platform.

experimental setup. The experiment was considered inappropriate for the collection if the experimental groups consisted of less than three biological replicates or if the experimental design introduced an unmanageable batch effect. Such batch effects were commonly introduced by consistently labeling different experimental groups with separate dyes in a two-color microarray experiment (i.e. lack of dye swapping). Furthermore, data sets representing non-commercial/custom or marginally represented platforms, for instance microarrays specific for miRNA or lncRNA, were excluded. As a result, only commercial gene expression microarrays from Agilent, Affymetrix, and Illumina were included alongside Illumina RNA-Seq platforms. The manual quality assessment of the collection is further described in the section *Technical Validation*.

Data preprocessing. Preprocessing of transcriptomics data must be performed prior to any further analysis. The current standard preprocessing pipeline for microarray data includes steps for sample quality checking, probe filtering, data normalization, batch effect assessment and correction as well as probe annotation[8]. Similarly, the state-of-the-art preprocessing of RNA-Seq data includes quality control, read alignment, read count extraction, filtering low counts, normalization, and batch effect assessment[8]. Here, each data set was preprocessed and analyzed individually. Data sets consisting of several cell lines or tissues were further separated by the biological system to better focus on the transcriptional differences between the exposures.

Preprocessing was performed in the R programming language (R version 3.5.2) following standard preprocessing pipelines suitable for each platform. For Agilent and Affymetrix microarrays, the preprocessing was implemented in the software eUTOPIA[9]. For Illumina BeadChips, a similar approach was applied following the suggested workflow of the R Bioconductor package lumi[10]. The preprocessing workflow applied to each platform is summarized in Fig. 2.

Quality check. Omics data are prone to technical errors that can arise from sample handling as well as the intrinsic characteristics of the platforms[8]. For this, an important step prior to any manipulation of the data is the quality check (QC) that allows the assessment of the gene expression distributions across samples revealing outliers and poor-quality samples. We applied a platform specific QC on each data set to evaluate the quality of the samples as well as the prevalence of outliers in the data.

For Agilent microarrays, the R package arrayQualityMetrics[11] was used, while the QC for Affymetrix was performed using the R packages affyQCreport[12] and yaqcaffy[13]. Outliers were further assessed based on the visual representation in the form of density plots, bar plots, dendrograms, and multi-dimensional scaling (MDS) plots, which were also the primary method of outlier detection for Illumina arrays. Outliers were removed from subsequent preprocessing and analysis.

Quality checking of the RNA sequencing data was performed using FastQC v0.11.7 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

Read alignment. RNA sequencing reads of mouse samples were aligned against the mouse reference genome assembly GRCm38, while sequencing reads of human samples were aligned against the human reference genome assembly GRCh38. The alignment was performed using the HISAT2 algorithm[14,15] employing the genome indexes built for usage with HISAT2 (retrieved from https://ccb.jhu.edu/software/hisat2/manual.shtml).

Sequencing file format conversions, such as.sam to.bam, sorting and extraction of uniquely mapped reads were performed using SAMtools (version 1.8-27-g0896262)[16].

Read counts extraction. Raw read counts for the RNA-Seq data were computed using the R package Rsubread (v2.2.3)[17]. The human Gencode version 35 annotation was applied for read counts extraction of human samples, while for mouse samples the mouse Gencode version M25 was employed. Both of the annotations were downloaded from https://www.gencodegenes.org.

Low counts filtering. In order to filter out the transcripts with low expression levels in the samples of each RNA-Seq dataset, the proportion test was used as implemented in the Bioconductor NOISeq package (v2.31.0)[18].

Probe filtering. For microarray experiments, probe filtering is commonly applied to remove probes showing low variance in the intensity range similar to the background[8]. These low-intensity probes were removed prior to data normalization. For Agilent microarrays, filtering was based on estimating the robustness of the probe signal intensities against the background (negative control probes) and applying a quantile-based method for eliminating probes with low signals. Individual thresholds based on the data and the number of experimental groups and replicates were determined for Agilent. For Illumina gene expression microarrays, probe filtering was performed after normalization based on the detection p-values[10] provided in the raw data. Only probes with a detection p-value < 0.01 in at least one sample were considered for further analysis.

Normalization. Normalization of transcriptomics data is crucial for robust comparisons of gene expression. Here, the normalization of the expression signal distribution in the samples was performed on the log2 transformed signal intensities using the quantile normalization from the R package limma[19] for Agilent, and the function *justRMA* from the package affy[20] for Affymetrix microarrays, respectively. For Illumina microarrays, quantile normalization was performed with the function *lumiN* from the lumi R package[10], while for Illumina RNA-Seq data, normalization was performed using the Bioconductor DESeq. 2 package[21]. In detail, the filtered raw counts underwent normalization by median of ratios method implemented in the package (for details see DESeq. 2 documentation).

Batch effect assessment and correction. Microarray experiments are susceptible to technical variation arising from the experimental setup, sample preparation, and the equipment, for example. This type of variation can lead to decreased quality and incorrect results. Thus, reducing the variation associated with technical variables (batch effect), while maintaining biological variation, improves the robustness of the results. Here, batch effects were evaluated by inspecting the results of principal component analysis, hierarchical clustering and multi-dimensional scaling[9]. Technical variation arising from unknown batches were evaluated with the function *sva* from the R package sva[22]. If variation associated to known technical variables or any of the surrogate variables was observed, its correlation with biological variables of interest was assessed via a confounding plot[23]. Batches that were not confounded with any of the variables of interest were corrected using the *ComBat*[24] function from the R package sva[22].

Probe annotation. Lastly, it is meaningful to map the probes to genes. For Agilent, the latest version of the annotation file for the specific microarray design was downloaded from the Agilent eArray website (https://earray.chem.agilent.com/earray/, 2020), and the probes were mapped to the Ensembl transcript IDs[25]. For Affymetrix gene expression arrays, the latest available alternative CDF files with Ensembl gene ID mappings were downloaded from Brainarray (http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/CDF_download.asp, 2020), while for Illumina BeadChips, the platform specific R annotation packages (illuminaHumanv3.db[26], illuminaHumanv4.db[27], illuminaRatv1.db[28] or illuminaMousev2.db[29]) were used.

Multiple probes mapped onto the same gene ID were summarized by their median values. Agilent probes that were initially annotated to Ensembl transcripts were further mapped to the corresponding Ensembl gene IDs. If multiple transcripts were mapped to the same gene, the one with the highest absolute score, as calculated by the $-log(p\text{-}value) \times log_2(fold\ change)$ for each exposure *vs*. control pairwise comparison, was selected.

Differential expression analysis. Transcriptomics analysis aims at identifying gene expression differences between biological conditions. Here, we performed a differential expression analysis on each microarray data set using the R package limma[19]. Comparisons were made between each specific experimental group consisting of a single exposure, dose, and time point and its corresponding control samples. Batch corrected variables were included as covariates of the linear model. In case the biological material was obtained from human donors, the donor was included as a covariate for the analysis. For RNA-Seq based data sets similar comparisons were made using the Bioconductor DESeq. 2 package[21].

As a result of the differential expression analysis, we provide full lists of genes with their specific fold changes and statistics as well as the results filtered to only contain significantly differentially expressed genes with the threshold of $|\log FC| > 0.58$ and Benjamini & Hochberg adjusted p-value < 0.05. Due to the implementation of DESeq. 2 independent filtering (for details see DESeq. 2 documentation), we also computed the adjusted p-values for RNA-Seq data externally from DESeq. 2 to obtain the full list of adjusted p-values with no missing values. These values are included in the unfiltered result files of the differential expression analysis under the column "adj.P.Val.no.ind.filt".

FAIRness optimization. To further assist accessibility, interoperability and reusability, the data sets have been curated, imported and made publicly available from the NanoPharos database (https://db.nanopharos.eu/), which has been developed under the Horizon 2020 (H2020) NanoSolveIT[30] (https://www.nanosolveit.eu) and

NanoCommons projects (https://nanocommons.eu/). The NanoPharos database has been primarily developed to include computationally derived data based on simulations for ENMs at different levels of accuracy. The database was then further extended to include ENM characterization data and biological effects. With the inclusion of omics data, the NanoPharos database is now covering, in a ready for modelling format, the full spectrum of data needed to initiate a computational workflow for in silico exploitation of the data. The data set was checked for inconsistencies in the data structure and harmonized where needed. The ENM physico-chemical characterization data have been enriched, where applicable, with molecular (*e.g.* atomic/ionic radii, electronegativity, energy band gap) and structural (*e.g.* crystallographic space group, unit cell dimensions and angles). Each ENM has been linked to the respective transcriptomics data set to facilitate querying and user study. The datasets can be queried and grouped, among others, based on the ENM core material, ENM batch, exposure time and dose, biological information, experiment type, analysis platform etc. (Supplementary File 1).

The NanoPharos database has been designed under the FAIR data principles[3] to offer users with high-quality, ready-for-modelling data sets, while allowing further development, adaptation and expansion. The FAIR data principles are meant to help database managers to improve data accessibility and reusability from the wider community in a way resembling Library Science[31]. To achieve this, data digitization in the NanoPharos database is being optimized to be machine readable to allow the seamless data comparison, transformation and, where possible, combination, providing the user with bigger and more complete data sets. On top of that, the NanoPharos database goes beyond the technical character of the FAIR data principles and is implementing the scientific FAIR data principles (SFAIR) as defined recently by Papadiamantis *et al.*[31], providing users with the necessary scientific context and background information for them to be able to reuse the data with the highest possible confidence. Furthermore, NanoPharos is readily accessible via Representational State Transfer (REST) application programming interface (API) and is able to interact with external databases (*e.g.* NanoSolveIT Cloud) and modelling tools through API programmatic access. The available datasets can be accessed through: https://db.nanopharos.eu/Queries.

Data Records

The data collection[32] generated here is freely available on Zenodo at https://doi.org/10.5281/zenodo.4146981. The collection comprises 85 preprocessed microarray-based data sets totaling 506 unique ENM vs. control comparisons and 16 RNA-Seq based data sets representing 23 ENM vs. control comparisons. Additionally, 24 comparisons of non-nanoparticle compounds used as positive/negative controls in the original experiments are included for the microarray data sets and 7 additional compounds are included for the RNA-Seq data. All of the data sets and their descriptions are available in Online-only Table 1, while the physico-chemical characteristics of the tested ENMs are available in Online-only Table 2, respectively.

In order to facilitate the selection of data suitable for different applications and modelling approaches, we classified the data into four categories based on the experimental design as follows:

I – Multiple doses, multiple time points.
II – Multiple doses, one time point.
III – One dose, multiple time points.
IV – One dose, one time point.

The proportion of each data class in the collection is visualized in Fig. 3a. Each class contains data obtained both *in vivo* and *in vitro* with at least two organisms represented (Fig. 3b). The collection covers a range of ENM compositions, as well as variants in size, shape, surface capping/coating etc. within a specific composition, in multiple biological systems in these organisms (Fig. 3c,d).

Files available for each data set. Each data set contains a homogenized metadata file, normalized and batch corrected expression matrices as well as complete and filtered results of the differential expression analysis (Table 1).

Technical Validation

The quality of transcriptomics data is a product of careful design of the experiment, technical execution as well as reporting of the data. The results of each downstream analysis substantially rely on the quality of the data. For this, we ensured that the collection contains high-quality data sets and defined a selection of criteria for data sets to be included:

- Three or more biological replicates are included for statistical robustness
- Microarray platform is a commercial gene expression microarray produced by Agilent, Affymetrix or Illumina
- The labelling of 2-color microarrays has been done considering dye swapping
- Non-normalized raw data is available
- Supporting information reports all variables required for preprocessing
- Untreated control samples are included

Each entry was evaluated based on the criteria, and either removed from the collection or selected for further preprocessing and analysis. The number of entries discarded for each of the listed reasons is represented in Table 2. Out of the 124 original entries 84 passed the quality assessment and were further divided into a total of 101 data sets (85 microarray and 16 RNA-Seq) based on the biological systems as specified in Data preprocessing.

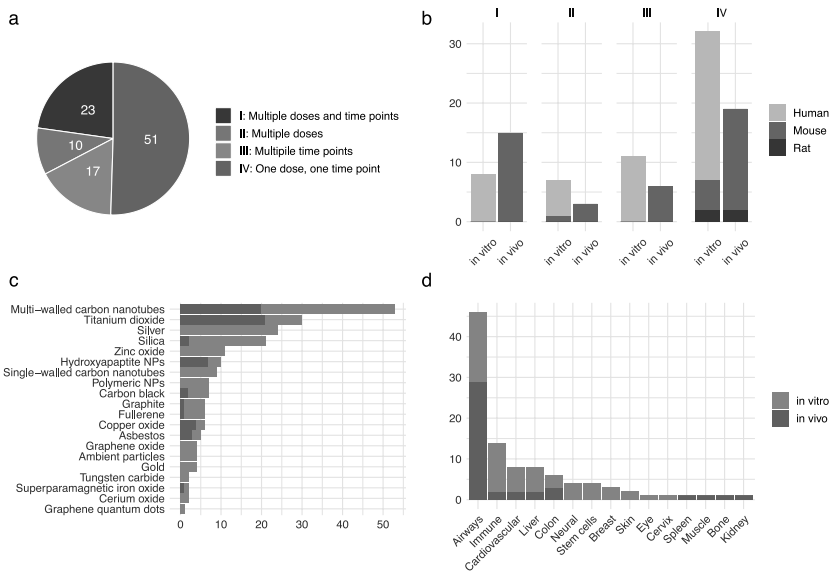

Fig. 3 The data collection comprises of various experimental setups and exposures of multiple ENM compositions. (**a**) The total of 101 data sets were divided into four classes based on the experimental setup. The pie chart represents the distribution of data sets by class. (**b**) Bars representing the proportion of data sets in each organism divided by the four classes. In vivo and in vitro exposures are separated. (**c**) Horizontal bars represent the number of data sets with the specific ENM core material or material type. Grey bars represent in vivo exposures and pink bars in vitro exposures, respectively. (**d**) Bars represent the biological system used in the experiment. In vitro exposures are represented by pink bars and in vivo exposures by grey bars.

Output file	File extension	Description
Metadata	txt	Sample information containing the following columns: *GSE, GSM, treatment, group, organism, biological_system, dose, dose_unit, time_point, time_point_unit, slide, array, dye, platform, filenames*, (and *donor*).
Normalized expression matrix	txt	Ensembl IDs as row names, sample IDs (GSM) as column names. Values are $\log_2$-transformed and normalized signal intensities resulting from the preprocessing for microarrays, and normalized read counts for RNA-Seq data, respectively.
Corrected expression matrix	txt	Ensembl IDs as row names, sample IDs (GSM) as column names. Values are $\log_2$-transformed, normalized, and batch corrected signal intensities for microarrays. Only included for microarray-based entries for which applicable.
Unfiltered differential expression results	xlsx	Excel file containing a sheet for each comparison (experimental group *vs.* control group) in the data set entry. Each sheet is named "*group-control*" and contains the following columns: *LogFC, AveExpr, t-statistic, P.value, adj.P.Val, B-statistic, score* and *ID*, as specified in the output of the limma R package[16] for microarrays. Columns available for RNA-Seq are *ID, baseMean, logFC, lfcSE, stat, P.Value, adj.P.Val* and *adj.P.Val.no.ind.filt*. Results contain all the genes in the platform after filtering and annotation.
Filtered differential expression results	xlsx	Excel file containing a sheet for each comparison with significantly differentially expressed genes with $\|logFC\| > 0.58$ and adj.P.Val < 0.05. Each sheet is named "*group-control*" and contains the following columns: *LogFC, AveExpr, t-statistic, P.value, adj.P.Val, B-statistic, score* and *ID*, as specified in the output of the limma R package[16] for microarrays. Columns available for RNA-Seq are *ID, baseMean, logFC, lfcSE, stat, P.Value* and *adj.P.Val*. Only included for entries for which significantly altered genes were found.

Table 1. Files provided for each entry in the collection.

Usage Notes

Here we provide the biggest homogenized collection of transcriptomics data sets in the field of nanosafety supplemented with metadata and ENM physico-chemical characteristics. The collection offers a valuable source for multiple analysis and modeling approaches[33]. For instance, the mechanism of action of each ENM can be characterized by investigating the provided lists of differentially expressed genes, and may be linked to specific physico-chemical characteristics such as size, surface capping or coating which can guide redesign of ENMs that

Reason to discard	Number of entries
Lack of replicates	26
Non-commercial or marginally represented platform	5
Two-color setup with no dye swapping	4
No raw data available	2
Incomplete metadata	2
Lack of control samples	1
Total entries discarded	40

Table 2. Reasons for discarding data during the manual quality assessment.

are safer and may support grouping into sets of nanoforms in accordance with REACH regulation (https://echa.europa.eu/documents/10162/13655/how_to_register_nano_en.pdf/f8c046ec-f60b-4349-492b-e915fd9e3ca0), for example. Moreover, pathway enrichment analysis can be performed to annotate these genes onto biological functions[34]. ENMs can be further compared and grouped based on the similarities between their molecular alteration profiles.

Due to the homogenized preprocessing and manual curation of the metadata, this collection is a relevant resource for identification of toxicity biomarkers. This can be addressed by using multiple feature selection approaches[35,36] or more advanced data modelling techniques[37–39]. Biomarkers could also be detected by means of gene co-expression network analysis, under the assumption that central network genes play a key role in the adaptation to the exposure[40,41].

The availability of data for multiple organisms or tissues can contribute to the development of more accurate adverse outcome pathways by linking ENM-specific molecular initiating events with cascades of relevant biological processes leading to an adverse outcome[42,43]. In addition, our data collection can be easily integrated with other transcriptomics data in the context of a read-across analysis to identify similarities in the molecular alterations induced by the ENMs with other phenotypic entities such as chemicals, drugs, and diseases[44]. Moreover, the data sets that we denoted as class I and II, where exposure at multiple doses are available, can be further analyzed to identify dose-dependent molecular alterations[45–48].

Our manually curated transcriptomics data collection with supporting ENM descriptions will have a high impact on the nanosafety community and can aid the development of new methodologies for nanomaterial safety assessment[2,8,30,33,43].

Code availability

Preprocessing of the data was performed on R version 3.5.2. The preprocessing of Agilent and Affymetrix expression data was performed using eUTOPIA[9], an R shiny software freely available on https://github.com/Greco-Lab/eUTOPIA. Custom scripts used for preprocessing of Illumina BeadChip and RNA sequencing data are available on GitHub on https://github.com/grecolab/Public_Nano.

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Author contributions

L.A.S. collected and organized the data, preprocessed and analyzed the microarray data and co-authored the manuscript. A.F. preprocessed and analyzed the RNA-Seq data and co-authored the manuscript. I.L. collected the ENM characteristics data and co-authored the manuscript. A.G.P. co-authored the manuscript and curated the data for the NanoPharos database. A.T. co-authored the manuscript and further developed the NanoPharos database to cover omics data. G.M. & A.A. co-authored the manuscript, organized the curation of the data and the development of NanoPharos database to cover omics data. A.S. defined the methodology, contributed to supervising the study and co-authored the manuscript. D.G. conceived and supervised the study and co-authored the manuscript.

Competing interests

The authors declare no competing interests.

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PUBLICATION II

Prospects and challenges for FAIR toxicogenomics data

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Prospects and challenges for FAIR toxicogenomics data

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ARISING FROM Nina Jeliazkova et al. *Nature Nanotechnology* https://doi.org/10.1038/s41565-021-00911-6 (2021)

The article by Jeliazkova at al.[1] recently published in this journal addresses the pivotal topic of data sharing and reuse in nanosafety. Current research in the field is highly multidisciplinary, as described also in the recent call for reporting standards for bio–nano experimental studies[2]. Hence, the application of the general FAIR (findable, accessible, interoperable and reusable) principles[3], although valid, might fall short when considering field-specific needs and requirements. This is especially true for toxicogenomics, in which additional challenges are posed by the articulated data analytics as well as the need to integrate multiple datasets to increase the statistical power and domain of applicability of the resulting predictive models. These limitations substantially affect the possibility of including toxicogenomics-based evidence in safe-by-design protocols as well as in regulatory hazard and risk decisions.

In our recent effort to curate publicly available transcriptomics data from exposures with engineered nanomaterials[4], we initially identified 124 datasets. However, although nearly all these datasets were published in peer-reviewed articles, the data quality assessment resulted in the exclusion of 35 datasets due to problems in their overall usability, rather than reusability. These problems were primarily related to the experimental design, which suggests that several toxicogenomics datasets published in peer-reviewed articles present substantial design flaws that jeopardize the validity of any results extrapolated from them and stresses the need to critically evaluate even data that have been FAIRified. In other words, reinforcing rigorous reporting of data does not automatically ensure quality, which should be addressed in the early phases of the experimental design. In fact, our curation also raised another concern: even datasets deposited in established databases could still be made (more) FAIR[5] as, despite the availability of mature standards for minimum reporting of omics experiments (for example, MIAME[6] and MINSEQE (http://fged.org/projects/minseqe/)) to aid data FAIRness, several aspects remain undocumented in toxicogenomics studies. According to community-accepted minimum reporting standards and the FAIR principles, the primary experimental variables are to be described (for example, exposure doses and times). However, when it comes to the preprocessing and analysis of toxicogenomics data, these minimum standards often result in poor (re) usability due to the lack of batch-effect description (that is, potential systematic effects caused by reagents, microarrays and so on)[7–9] and incomplete characterization of the experimental design and execution[7]. This, in turn, prevents optimal data preprocessing and analysis, but could be easily overcome through additional criteria and quality checks built into the study design and reported as part of the required metadata.

Moreover, the reliance on minimum standards over complete documentation is not just a concern for the reuse of raw omics data. Similar challenges exist regarding the analysis and modelling performed on these data, which include the identification of predictive biomarkers, the development of adverse outcome pathways or the performance of the meta-analysis. Although the complexity of toxicogenomics data requires the use of articulated multistep analytical pipelines, their high dimensionality dictates the tailoring of algorithms and parameters to fit the specific characteristics of each experimental design and dataset. This has a profound impact, as equally technically valid alternative analytical strategies can lead to apparently divergent sets of results. Omics data analysis traditionally results in long lists of molecules that distinguish the experimental conditions assayed. These are intrinsically difficult to interpret unless functional analysis is performed to pinpoint over-represented biological functions. As the association of individual molecules with biological functions is, per se, an interpretative exercise, it is intuitive that alternative analytical strategies, which may result in slightly different sets of candidate molecules, may have a considerable impact on the interpretation of the final outcome. Indeed, this is one of the main reasons why toxicogenomics data still struggles to be fully accepted for regulatory purposes. Thus, ensuring the FAIRness of the computational protocols, tools and algorithms used to analyze toxicogenomics data can provide a sensible way to alleviate this bottleneck. In this regard, we advocate the need to differentiate between technical and scientific FAIRness[10]. Although the former can be addressed by sharing code, scripts and software to replicate a specific analysis, the latter focuses on the generation and sharing of standard operating procedures in which each analytical step is carefully motivated and described (metadata). Both technical and scientific FAIRness are equally important, albeit with slightly different 'owners' responsible for their implementation, and as a community we should define specific scientific FAIR principles for each of the different subdomains of nanosafety.

Finally, data curation is needed to advance research in many fields of modern science, and recognition of this huge effort is essential. Acknowledgement of the data generation effort is easily achieved through the publication of original research articles. However, curation of already published data often remains a sterile exercise in which the curated data, with increased FAIRness scores, remain fully available only to a small community of scientists. We propose two solutions to be adopted by authors and publishers, respectively. The former should consider curation as a valuable contribution to the field, and as such should publish the curated dataset and the associated curation protocols in one of

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the myriad of data-focused journals. Publishers can contribute by requiring the bulk of the curated data that underpins meta-analyses and chemo- and nanoinformatics models to be accessible via well-established data repositories (such as Zenodo), via specific open curation databases (for example, the NanoPharos Database (https://db.nanopharos.eu/Queries/Datasets.zul)) and/or via other database platforms. Reuse of curated data will be facilitated by ensuring that the data are exported in formats that are suitable for modelling or further analysis.

With these considerations in mind, we believe that it is meaningful to address the overall usability of published data in addition to the aspects of FAIR, and that the usability can be improved through many of the actions already suggested by the nanosafety community[1,2,5,7–11]. The challenges discussed in this comment are not unique to nanosafety but pervade the toxicogenomics field as a whole. However, notable efforts, such as that by Jeliazkova et al.[1], place the nanosafety community at the forefront of advancing the entire area of chemical safety assessment. Indeed, the nanosafety community is driving the updating of regulatory testing on a wide scale. Supplementing the broad technical FAIR principles with subdomain-specific considerations, as represented here by the toxicogenomics field, will considerably increase the transparency of results and predictions based on the reuse of such data. Furthermore, it will pave the way towards regulatory acceptance of toxicogenomics-based evidence in the safety assessment of engineered nanomaterials and other chemicals alike.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41565-021-01049-1.

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Author contributions
L.A.S. carried out the formal analysis, contributed to the data curation and methodology, and co-wrote the original draft. G.M. contributed to the methodology, review and editing. A.A. contributed to the methodology, review and editing, and funding acquisition. I.L. contributed to the data curation and funding acquisition, and co-wrote the original draft. D.G. conceptualized and supervised the work, contributed to the funding acquisition and co-wrote the original draft.

Competing interests
The authors declare no competing interests.

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PUBLICATION III

Toxicogenomics analysis of dynamic dose-response in macrophages highlights molecular alterations relevant for multi-walled carbon nanotube-induced lung fibrosis

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Toxicogenomics analysis of dynamic dose-response in macrophages highlights molecular alterations relevant for multi-walled carbon nanotube-induced lung fibrosis

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ABSTRACT

Toxicogenomics approaches are increasingly used to gain mechanistic insight into the toxicity of engineered nanomaterials (ENMs). These emerging technologies have been shown to aid the translation of *in vitro* experimentation into relevant information on real-life exposures. Furthermore, integrating multiple layers of molecular alteration can provide a broader understanding of the toxicological insult. While there is growing evidence of the immunotoxic effects of several ENMs, the mechanisms are less characterized, and the dynamics of the molecular adaptation of the immune cells are still largely unknown.

Here, we hypothesized that a multi-omics investigation of dynamic dose-dependent (DDD) molecular alterations could be used to retrieve relevant information concerning possible long-term consequences of the exposure. To this end, we applied this approach on a model of human macrophages to investigate the effects of rigid multi-walled carbon nanotubes (rCNTs). THP-1 macrophages were exposed to increasing concentrations of rCNTs and the genome-wide transcription and gene promoter methylation were assessed at three consecutive time points. The results suggest dynamic molecular adaptation with a rapid response in the gene expression and contribution of DNA methylation in the long-term adaptation. Moreover, our analytical approach is able to highlight patterns of molecular alteration *in vitro* that are relevant for the pathogenesis of pulmonary fibrosis, a known long-term effect of rCNTs exposure *in vivo*.

1. Introduction

The immunotoxic potential of multi-walled carbon nanotubes (MWCNTs) on the respiratory system has already been reported with the support of toxicogenomics evidence (Halappanavar et al., 2019; Labib et al., 2016; Kinaret et al., 2017a, 2017b; Poulsen et al., 2017; Rydman et al., 2014). Furthermore, we have recently demonstrated that analyzing the transcriptome from *in vitro* as well as *in vivo* exposures can successfully inform on relevant patterns of molecular adaptation, possible toxic outcomes, and inflammatory responses (Kinaret et al., 2017a). While the use of toxicogenomics in the assessment of adverse effects of chemical exposures is gaining acceptance, chemical risk assessment still largely relies on expensive and laborious animal experiments. *In vitro* models are increasingly used in compliance with the 3R principles (Replacement, Reduction and Refinement) of animal experimentation (Russell and Burch, 1959). Although long-term effects have been investigated *in vitro* [*e.g.* (Comfort et al., 2014; He et al., 2016; Holmgren et al., 2014; Luanpitpong et al., 2014; Wang et al., 2011)], they typically require weeks to months of continuous exposure, hardly cutting back on the time and effort. While recent developments in toxicogenomics can support more advanced interpretation of toxic mechanisms also from *in vitro* models, we are still lacking robust methods for the interpretation of potential long-term effects from short-term assays.

Alterations in DNA methylation have been suggested as a mechanism

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of more persistent molecular changes upon engineered nanomaterials (ENMs) exposures (Brown et al., 2016; Öner et al., 2017; Scala et al., 2018a; Stapleton et al., 2018; Sierra et al., 2017). More recently, multi-omics approaches have been used to explain the complex patterns of molecular adaptation to ENMs in multiple cell types (Scala et al., 2018a). Hence, we hypothesize that relevant information on possible long-term effects of MWCNT can be obtained in a short *in vitro* exposure set-up, by combining the analysis of dynamic gene expression changes with epigenetic alterations that are more stable by nature.

The airways are one of the most prominent routes of exposure to ENMs, making resident lung cell types a valid model for assessing the toxicity of ENMs *in vitro (*Kinaret et al., 2017a*;* Scala et al., 2018a*;* Stoccoro et al., 2017*;* Søs Poulsen et al., 2013*)*. Resident macrophages, together with neutrophils, represent the first line of defense against pathogens and foreign bodies introduced into the airways (Farrera and Fadeel, 2015). These phagocytic cells attempt to internalize the foreign intruders initiating defense mechanisms that can lead to inflammation and, further, damage the tissue when unresolved. Many ENMs are, however, rather biodurable, making their enzymatic degradation a difficult task for phagocytes (Liu et al., 2010; Vlasova et al., 2016). Furthermore, many rigid fiber-like particles are difficult to engulf due to their high aspect-ratio, often leading to impaired ingestion and frustrated phagocytosis that further aggravates inflammation and contributes to the adverse consequences of ENMs exposures (Boyles et al., 2015a; Murphy et al., 2012; Rydman et al., 2014). Moreover, recent evidence suggests that ENMs can impact macrophage polarization, thus affecting the long-term outcome of the exposure (Dong and Ma, 2018; Meng et al., 2015; Kinaret et al., 2020). Although the role of macrophages in reactions induced by carbon nanotubes (CNTs) is recognized, the dynamic patterns of molecular adaptation of macrophages to MWCNTs are yet to be discovered (Kinaret et al., 2017b; Rydman et al., 2014).

To date, dose-dependent molecular responses have been investigated by focusing on individual time points of exposure (Bourdon et al., 2013; Labib et al., 2016). This approach, however, makes the interpretation of the kinetics of molecular adaptation difficult. To overcome this limitation, we applied a novel computational approach that allows the modelling of dynamic dose-dependent (DDD) alterations in two distinct molecular layers, the transcriptome and the DNA methylome (Serra et al., 2020). We applied this analytical strategy in order to model a robust dynamic mechanism of action of MWCNTs on a human macrophage cell model. We exposed macrophage-like cells derived from the human monocytic THP-1 cell line to three different doses (5, 10 and 20 μg/mL) of long and rigid multi-walled carbon nanotubes (rCNTs) for three consecutive time points: 24, 48, and 72 h. We then investigated the DDD molecular alterations at the level of the transcriptome and gene promoter methylation. In this way, we were able to underline key molecular changes already described in lung fibrosis *in vivo*. Our approach supports the use of toxicogenomics in building more comprehensive predictions about the long-term effects of ENMs exposure, such as fibrosis, by using an *in vitro* exposure setup.

2. Material and methods

2.1. Nanomaterial

The multi-walled carbon nanotubes used in this study have been previously characterized in Rydman et al. and Kinaret et al. (Kinaret et al., 2017b; Rydman et al., 2015*)*. The properties of the nanomaterial are summarized in Table 1.

2.2. Cell culture and exposures

THP-1 cells (DSMZ ACC 16) were cultured at 37 °C in cell culture flasks in RPMI 1640 media (Gibco, Thermo Fisher Scientific, USA) with 10% FBS (Gibco), 2 mM ultraglutamine (Gibco), and 1% penicillin-streptomycin (Gibco) supplementation (complete RPMI media). Cells were plated into six-well plates ($1.0 \times 10\hat{}6$ cells/well) and differentiated for 48 h with 50 nM PMA (phorbol 12-myristate 13-acetate, Merck KGaA, Darmstadt, Germany). Fresh, complete RPMI media with PMA was replaced after 24 h and after a total of 48 h, fresh complete media without PMA was added. The control group was treated in a similar manner, with complete media and PMA, without the rCNTs.

Dispersion of the rCNTs was based on publications by Bihari et al. (Bihari et al., 2008) and Gallud et al. (Gallud et al., 2020), and successfully used in several previous publications by us and others (Boyles et al., 2015b; Chortarea et al., 2019; Kinaret et al., 2017a; Kinaret et al., 2020; Scala et al., 2018a). A stock solution of 1 mg/mL of rCNTs was freshly prepared prior to exposure in a sterile glass tube with plain RPMI 1640 media, vortexed for 1 min and sonicated in a bath sonicator (37 kHz, Elmasonic S30 (H), Ilabequipment, USA) for 3×15 min at room temperature. The stock solution was then diluted with complete RPMI media to obtain final exposure concentrations of 5, 10, and 20 μg of rCNT/mL (corresponding to mass per area exposure concentrations of 1.04, 2.08 and 4.16 $\mu g/cm^2$ as a total volume of 2 ml of exposure media was used). The dose range was selected to reflect low-dose exposures that are high enough to induce a response without showing significant cytotoxicity (Scala et al., 2018a). The dilutions were vortexed and sonicated prior to exposures for additional 15 mins. Cells (passage number P8) were exposed to rCNTs in complete RPMI for 24, 48 or 72 h. Cells treated in a similar manner, sonicated and RPMI media vortexed in the same way but without rCNTs exposure were used as a reference group. A total of 6 replicates of each treatment and dose pair were performed. After exposures, two replicates were pooled into one sample to produce a total of 3 independent replicates for each exposure group. These triplicates were then used for the following transcriptomics and DNA methylation experiments.

2.3. RNA/DNA extraction

Following each exposure period, the cells were harvested, and lysed, and total RNA was isolated using RNeasy Mini kit (Qiagen, Germany) following the instructions of the manufacturer. Total RNA samples were quantified with NanoDrop (ND-1000, Thermo Fisher Scientific) and the quality of the RNA was further verified using FragmentAnalyzer (Agilent Technologies, USA). RNA samples with RNA quality number (RQN) higher than 9.5 were used for the analysis.

DNA was extracted from the cell lysates using Maxwell® RSC Cultured Cells DNA Kit and the Maxwell® RSC instrument according to manufacturer's instructions (Promega Corporation, USA). Integrity of the DNA was verified by gel electrophoresis on 1% precasted *E*-gel (Invitrogen, Thermo Fisher Scientific) and quantified using PicoGreen (Quant-iT Broad-Range dsDNA Assay Kit, Invitrogen).

Table 1
Characteristics of the multi-walled carbon nanotubes used in the experiment.

Description	Product code	Provider	Aspect ratio	Average length [nm]	Average diameter [nm]	Average surface area [m^2/g]
Rigid multi-walled carbon nanotube	XNRI-7 mitsui	Mitsui & Co., Ltd. (Japan)	260	13,000	50	22

2.4. DNA microarrays

Total RNA samples (100 ng) were labeled and amplified using the T7 RNA polymerase amplification method (Low Input Quick Amp Labeling Kit, Agilent Technologies) following the instructions of the manufacturer (Agilent Technologies). cRNA samples labeled with either Cy3 or Cy5 fluorescent labels (Agilent Technologies) were purified with RNeasy Mini Kit (Qiagen). The quantity and specific activity of the samples were verified using NanoDrop (ND-2000, Thermo Fisher Scientific). Finally, 300 ng of cRNA labeled with Cy3 were combined with a corresponding amount of cRNA labeled with Cy5, fragmented, and hybridized onto the Agilent SurePrint G3 Human GE 8 × 60 microarrays. After washing, the slides were scanned with Agilent microarray scanner model G2505C (Agilent Technologies). Data were extracted using the Agilent Feature Extraction software (V12.0.2.2). Microarray data have been submitted to NCBI Gene Expression Omnibus (GEO) database under the series accession number GSE146710.

2.5. Genome-wide DNA methylation

Genome-wide DNA methylation analysis was performed using the Infinium HD methylation assay (Illumina, USA) according to manufacturer's protocol. First, DNA samples (500 ng) were bisulfite converted using the EZ-96 Methylation Kit (Zymo Research, USA) following the instructions of the manufacturer. Next, DNA was amplified, fragmented, and hybridized to the Infinium MethylationEPIC BeadChips, and finally, scanned with the iScan scanner (Illumina).

2.6. Transcriptomics

Transcriptomics data were preprocessed using the R Shiny application eUTOPIA (Marwah et al., 2019). Raw data were imported, and low-quality probes were filtered out using a quantile-based approach. Particularly, probes with a value higher than 75% quantile of negative probes in at least 85% of the samples were selected for further steps. The log2 transformed intensity values were then normalized between arrays using quantile normalization. Technical variation resulting from the dye, slide, and the position on the slide were eliminated by batch correction with the ComBat method from the R package sva (Leek et al., 2012). Finally, multiple probes mapped to the same gene symbol were summarized by their median values.

Differential expression between each exposure group (one dose at one time point) and their corresponding control group was estimated by linear models followed by empirical Bayes pairwise comparison as implemented in the R package limma (Ritchie et al., 2015). Corrected batches were included as covariates in the analysis. Genes with a fold change $>|1.5|$ and Benjamini & Hochberg adjusted $p < 0.05$ were considered significantly differentially expressed.

2.7. CpG methylation

Methylation data were preprocessed using eUTOPIA following the workflow of the application (Marwah et al., 2019). Raw methylation files were uploaded together with the phenotype file. CpG probes were filtered by removing probes with a detection p-value >0.01 in any sample. Further filtering was applied to remove probes for CpGs located on the sex chromosomes, those containing single nucleotide polymorphisms or belonging to a set of known cross-hybridizing probes (Chen et al., 2013). Data were normalized using the Subset-quantile Within Array Normalization (SWAN) method (Maksimovic et al., 2012). Batch correction was performed with the Combat method from the R package sva to remove technical variation associated to the chip (Leek et al., 2012). Finally, a gene promoter region was defined as 200 bp upstream from the transcription start site of each gene, and the M-values for CpG probes in the promoter region were summarized by their median value for each gene. M-values were transformed into Beta-values using the function m2beta from the R package lumi (Du et al., 2008).

Differential methylation analysis was performed with the limma approach as described above for transcriptomics (Ritchie et al., 2015). The gene promoter was considered significantly differentially methylated with a fold change $>|1.2|$ and $p < 0.01$.

2.8. Dose- and time modelling

Each molecular layer was analyzed separately with parallel approaches following the workflow of TinderMIX (Serra et al., 2020). In brief, sample-wise fold changes were calculated between each exposed sample and each of its corresponding control samples. Fold-changes were log2 transformed and used for the modelling. For transcriptomics data, two-way ANOVA was applied to identify the genes whose fold change showed variance significantly ($p < 0.01$) associated to dose, time, or the interaction of dose and time. These genes were considered "responsive" and selected for further modelling in both molecular layers. First, a selection of polynomial models (linear, second and third order) were fitted to the known points. The optimal model for each gene was selected based on the lowest Akaike Information Criterion (AIC) value, and the genes with a non-significant p-value (FDR corrected $p > 0.05$) for the fitting were filtered out. Afterwards, the dose and time ranges were divided into 50 equally distributed bins, and the optimal model of each gene was used to predict their corresponding log2 fold changes. In such a way, each gene is represented by a 50 by 50 activation map in the space of time and dose, that is able to interpolate the doses and time points not included in the experiment. From each activation map, an area with monotonically increasing or decreasing (with respect to the dose) fold change greater than the activity threshold (fold change$>|1.1|$) was recognized by means of its gradient matrix and determined as "responsive area". If such an area could be identified, the gene was considered to be altered in dynamic dose-responsive manner. Finally, the activation map was divided into three equal sections on the time axis and each section was assigned a label: "early", "middle", and "late", respectively, according to the implementation of the TinderMIX software (Serra et al., 2020). With this approach, each gene was assigned one of these labels based on the time of activation of the gene, *i.e.* the section in which the activity threshold was surpassed at the earliest time point and the lowest possible dose.

2.9. Functional enrichment

Pathway enrichments were performed using an R-shiny graphical tool FunMappOne (Scala et al., 2019). Lists of official gene symbols were offered as an input with the direction of the alteration (fold-change increasing or decreasing with dose) as a modification of the genes. Reactome annotations were used for pathway enrichment, and all known genes were used as the statistical domain scope of the analysis. Pathways were considered significantly enriched with a p-value <0.01 adjusted with the g:SCS method (Reimand et al., 2007).

3. Results and discussion

Given the important role of macrophages in the systemic responses to ENMs exposure, we decided to focus on a macrophage *in vitro* model. We aimed at disentangling the kinetics of the molecular adaptation by using a multi-omics approach in combination with multiple time points and rCNTs doses showing low toxicity (Scala et al., 2018a).

3.1. Transcriptional changes follow a dose-dependent trend

Differential expression is often used to statistically evaluate the quantitative transcriptomic changes between experimental groups. Exposing macrophages to increasing concentrations of rCNTs for three consecutive time points resulted in a total of 5495 differentially

expressed genes (DEGs) (Table S1). A clear dose-dependent increase in the number of DEGs can be visually observed at each time point, suggesting a more impactful exposure as the dose increases (Fig. 1A). On the contrary, the number of differentially methylated promoters (DMPs) only showed a visually increasing dose-dependent trend at 24 h and a dose-dependently decreasing number of hypomethylated promoters was observed at 48 h (Fig. 1B). Total of 307 gene promoter regions showed significant differential methylation as compared to unexposed controls. The subtle impact on DNA methylation observed in the present study is in line with previous reports suggesting limited changes of the methylation levels in fewer *loci* upon exposure to MWCNTs (Öner et al., 2017; Scala et al., 2018a; Sierra et al., 2017). However, we observed a convincing pattern of DNA methylation adaptation at the highest dose and the longest exposure time with a higher prevalence of hypomethylated promoters (Fig. 1B). While the gene expression is regulated by dynamic mechanisms, such as transcription factor binding, regulation of the enzymatic machineries controlling DNA methylation is slower and also involves cell replication -dependent events (Edwards et al., 2017). For this reason, we cannot exclude that some of the regulatory mechanisms of DNA methylation are not active in a differentiated cell type/cell culture, where cell cycle is largely halted. This might be progressively evident as the exposure time proceeds through the 72 h.

3.2. Dynamic dose-dependent analysis highlights an additional set of adaptive genes

The conventional analysis of differential expression/methylation by comparing treated to non-treated samples follows the observations of several previous studies indicating a prominent transcriptomic response but a marginal impact on the DNA methylation (Kinaret et al., 2017a; Öner et al., 2017; Scala et al., 2018a; Sierra et al., 2017). We suspected that part of the molecular effects are directly induced by the chemical agents and substances and thus, show dose-dependent behavior. Hence, we focused on finding the genes whose behavior is monotonically altered with an increasing dose. Benchmark dose (BMD) modelling has been proposed to identify such genes (Yang et al., 2007). Although BMD modelling succeeds in highlighting monotonically altered genes, it gives little insight into the kinetics of the molecular alteration. Here, we investigated the DDD behavior of the genes upon rCNTs exposure by simultaneously modelling the effects of the dose and time. With this integrated approach, we obtained a total of 6428 genes with DDD alteration in gene expression and 414 genes with DDD changes in the promoter methylation. The genes with DDD behavior were labeled "early", "middle", or "late" based on their point of departure (POD) in each distinct molecular layer (Table 2, Table S2). Interestingly, the kinetics of the molecular alterations showed distinct patterns in each molecular layer, as the majority of the changes in gene expression were initiated early, while alterations in the promoter methylation were mostly observed at late exposure time (Table 2). Given the more stable, regulatory nature of DNA methylation, later activation is expected. Evidence of later changes in DNA methylation has also been shown *in vivo*, as significant changes in DNA methylation levels in the lungs of mice exposed to MWCNTs were observed 7 days after the exposure, but not at 24 h (Brown et al., 2016).

Table 2
Number of dynamic dose-dependent molecular alterations obtained for gene expression and gene promoter regions, specified by their time of activation.

Activation time/Molecular layer	Early	Middle	Late	Total
Expression	3912	1092	1424	6428
Methylation	131	44	239	414

By comparing the differentially expressed genes and differentially methylated promoters with the respective set of DDD genes, we found an intersection of 3212 (58%) genes for expression (Fig. 2A), and an intersection of 70 (23%) promoters for methylation (Fig. 2B), respectively. As we suspected, these results suggest that the mechanism of action of rCNTs is only partially dose-dependent, with 58% of DEGs showing dose-dependent behavior, while molecular adaptation as a whole is achieved, not surprisingly, by complex circuits of non-monotonic molecular regulation. Furthermore, our approach identified a large proportion of molecular alterations not captured by the traditional differential expression/methylation analysis (Fig. 2A-B). Our integrated dose- and time- modelling approach allows us to retrieve also genes whose magnitude of alteration could not be sufficiently evident in each exposed *vs* control pairwise comparison. Furthermore, the investigation of the POD is especially useful in the regulatory setting for example in defining toxicological reference doses (Labib et al., 2016; Webster et al., 2015).

3.3. A proportion of dynamic dose-dependent genes are coupled with dose-dependent alteration also in the gene promoter methylation

The assessment of alterations in DNA methylation can be useful for predicting long-term effects of short-term exposures (Canzler et al., 2020). In order to understand the relationship between transcriptional and epigenetic adaptation, we investigated the two molecular layers in relation to each other. In total, the two layers shared 220 DDD genes (3.4% of all the DDD transcriptionally altered genes) (Fig. 3A, Table S3). These results are in line with our previous findings suggesting that only a proportion of genes acquire "more stable" molecular alteration in the form of DNA methylation (Scala et al., 2018a). When considering the

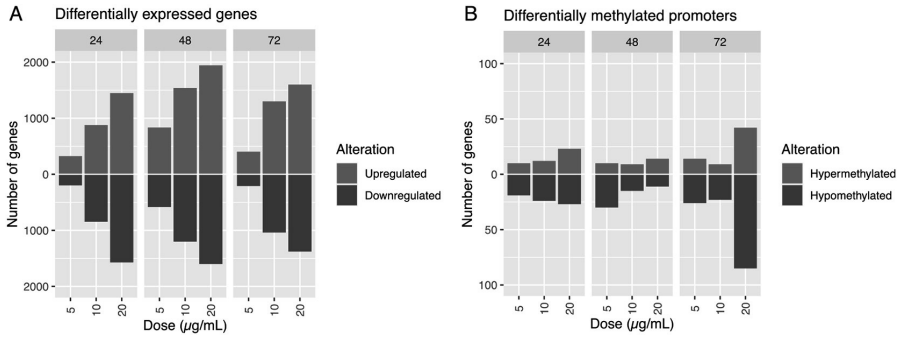

Fig. 1. Number of genes altered in respect to rCNTs exposure. Timepoints 24, 48 and 72 h of exposure with 5, 10 and 20 μg/mL exposure concentrations (Dose). (A) Differentially expressed genes with bars representing number of up- (red) and down- (green) regulated genes for expression (FC > |1.5|, FDR-corrected *p*-value <0.05) (B) and hyper- (red) and hypomethylated (green) promoters for differential methylation (FC > |1.2|, *p*-value <0.01), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

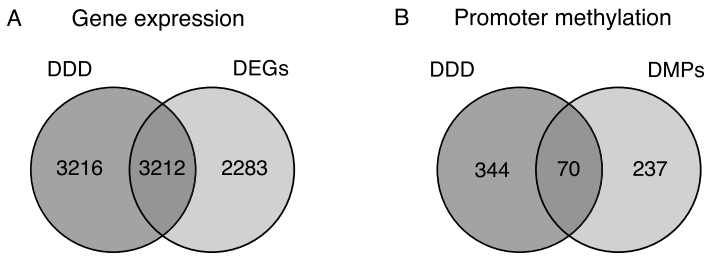

Fig. 2. Venn diagrams representing the overlap of genes obtained through the modelling of dynamic dose-dependent (DDD) alterations and standard analysis of differentially expressed genes (DEGs) (A) or differentially methylated promoters (DMPs) (B).

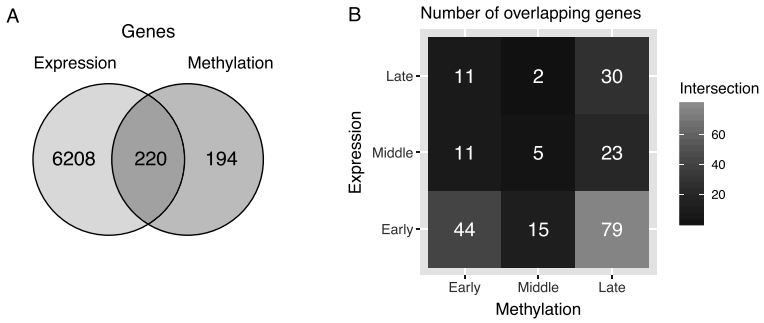

Fig. 3. Intersections between gene expression and promoter methylation at the level of all DDD genes (A) and the overlapping DDD genes and promoter regions grouped by activation time (B).

kinetics of these molecular alterations, we found the largest overlap (79 genes) between genes whose expression alteration was initiated early while methylation perturbation was triggered late, suggesting the role of DNA methylation in sustaining or repressing these expression patterns long-term (Fig. 3B).

The 220 common genes indicate several important macrophage functions to be affected by epigenetic regulation in response to rCNTs. Among the set of common genes, in fact, we identified multiple chemokine encoding genes that have a pivotal role in macrophage induced inflammation (Table S3). For instance, CXCL8, CXCL10, and CCL20 were upregulated, while their promoters were hypomethylated, suggesting a sustained long-term induction of these genes. We also observed altered behavior of several genes indicating calcium homeostasis, as the transcription of the calcium channel genes CACFD1, CACNA2D1, CACNA2D4, CACNG2 was repressed, while their promoters were generally hypermethylated. Downregulation of genes encoding for calcium channels could indicate an increased level of cytosolic calcium, which in turn triggers multiple signaling pathways in activated macrophages, including IL-1β secretion and the activation of NLRP3 inflammasome (Feske et al., 2015; Rada et al., 2014; Zumerle et al., 2019). Indeed, nano-sized particles have been shown to modulate intracellular calcium concentration, and exposure to long and rigid MWCNTs has been previously associated with NLRP3 inflammasome activation (Brown et al., 2004; Li et al., 2017; Palomäki et al., 2011). Both of these events are reported to be dependent on reactive oxygen species (ROS) production, which is also known to be acutely induced in our experimental setup (Scala et al., 2018a). Taken together, these results suggest the role of methylation in sustaining selected patterns of transcriptional adaptation of macrophages to rCNTs exposure.

3.4. DDD alterations in transcription and methylation are related to cell activation and homeostasis

To better understand the functionality of the DDD genes in each molecular layer, we investigated the enriched pathways in each group. Our analysis highlighted a total of 493 significantly enriched Reactome pathways for gene expression and 68 pathways for promoter methylation. Out of these, 63 pathways (13%) were shared between the two molecular layers (Fig. 4A). All the common enriched pathways were activated early in the transcriptome with a great proportion (68%) of them sustaining the enrichment along the time (Fig. 4B). Pathways such as "immune system", "disease", "signal transduction", "metabolism" and "cell cycle" represent many of the functions already highlighted by earlier toxicogenomic studies both in vitro and in vivo (Kinaret et al., 2017a, 2017b; Öner et al., 2017; Poulsen et al., 2017; Scala et al., 2018a).

We found several key pathways of the immune functions to be prominently represented, including well known responses of macrophages to MWCNTs exposure, such as *innate immune response*, *inflammation*, *cytokine signaling*, and *antigen processing and presentation* (Fig. 4B) (Kinaret et al., 2017a, 2017b; Poulsen et al., 2017; Scala et al., 2018a). Previous studies have also reported epigenetic regulation of these processes in response to MWCNTs (Öner et al., 2017; Scala et al., 2018a). Interestingly, in methylation, *immune system* was the only pathway already enriched at "middle" and sustaining it at "late", suggesting a more pronounced role of promoter methylation in the regulation of immune-related genes. The combination of alterations in transcription and promoter methylation suggests long-term regulation of the genes of these pathways. While the enrichment of immune related functions is not surprising considering the nature of the exposure, it indicates that our analytical approach is able to highlight short- and possible long-term patterns relevant for the exposure. In addition, several compartments of the cell metabolism were found to be altered, including *response to stress*

Fig. 4. (A) Venn diagram representing the intersection of all enriched Reactome pathways for genes with DDD expression and promoter regions with DDD methylation. (B) Heatmap representing the enrichment patterns of the 63 Reactome pathways common to gene expression and promoter methylation.

as well as *protein and RNA metabolic processes* (Fig. 4B). Alteration of general metabolic pathways could indicate cellular stress, while functions related to protein metabolism may support well known macrophage functions, such as antigen processing and presentation. The role of epigenetic alteration in protein metabolic processes observed in this study, have been also reported previously (Öner et al., 2017; Scala et al., 2018b).

The most represented common functions in this study were related to signaling pathways. We have previously observed a pronounced positive association between the alteration of intracellular signaling pathways and the nanoparticle aspect ratio (Scala et al., 2018a). Similar patterns of alterations have been reported at the level of the transcriptome *in vivo* as well as DNA methylation *in vitro* (Kinaret et al., 2017a, 2017b; Öner et al., 2017; Scala et al., 2018a). Here, our results highlight the activation of *MAPK signaling* pathways, as also previously reported (Kinaret et al., 2017a; Öner et al., 2017; Scala et al., 2018a). MAPK signaling has an important role in regulating innate immune responses as well as cell survival (Arthur and Ley, 2013; Cargnello and Roux, 2011). Interestingly, functions related to epigenetic regulation of gene expression were also retrieved among the pathways commonly represented in both data layers analyzed in this study, supporting the role of DNA methylation and its regulation in the adaptation of macrophages to rCNTs exposure.

3.5. Pathways underlying short-term adaptation are not coupled with promoter methylation changes

When looking at the alterations seen only at the level of the transcriptome, we observed that 79% of the pathways were initiated early and not sustained through time, indicating a rapid short-term macrophage response through transcriptomic alterations (Table S4). As in the common pathways between expression and methylation, immune functions, signaling pathways, and metabolic functions were highly represented in the DDD genes in the transcriptomics layer (Table S4). This suggests that epigenetic regulation of these functions is limited to a small number of genes needed for long-term adaptation. Furthermore, pathways observed only in the transcriptome include acute effects such as apoptosis and DNA damage response indicating a stress response that requires rapid engagement. Even at the sub-toxic concentrations used in the present study, rCNTs exposure is known to exert cellular stress, also observed here by the induction of apoptotic pathways, downregulation of cellular metabolism, and activation of DNA repair pathways (Kinaret et al., 2017a; Scala et al., 2018a; Srivastava et al., 2011).

Furthermore, we identified nuclear factor kappa B (NF-κB) signaling in the center of the macrophage transcriptomic response to rCNTs. NF-κB transcription factors rapidly regulate a wide array of genes involved in immune functions and inflammation, and NF-κB signaling can be activated by various *stimuli* (Liu et al., 2017). The canonical NF-κB signaling pathway activation is associated to several proinflammatory cytokines and pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs). Instead, the non-canonical pathway is generally activated through tumor necrosis factor (TNF) receptor superfamily members (Liu et al., 2017; Sun, 2017). Mukherjee et al. recently suggested NF-κB signaling as a central regulator of transcriptional responses to single-walled carbon nanotubes (SWCNTs) *via* a direct interaction with TLRs (Mukherjee et al., 2018). Of note, while TLR pathways are consistently found to be enriched in response to MWCNTs exposure, including this study (Table S4), direct physical interaction is reported in literature only between SWCNTs and TLRs (Mozolewska et al., 2015; Mukherjee et al., 2018). Considering the bigger diameter of MWCNTs particles (up to 50 times larger than SWCNTs), the interaction between different sized and shaped CNTs and TLRs might differ. In contrast to the canonical NF-κB signaling induced by SWCNTs, our study highlights early activation of *TNFR2 non-canonical NF-kB pathway* (Table S4), suggesting distinct modes of NF-κB signaling activation between different types of CNTs. Furthermore, our analysis showed an early induction of key genes of the non-canonical NF-κB signaling, including both subunits of the p52/RelB complex alongside several possible initiating molecules (*e.g.* CD40 and TNF), as well as the central signaling component of the non-canonical NF-κB pathway, MAP3K14 (Table S2) (Sun, 2017).

3.6. Macrophage molecular adaptation to rCNTs comprises alterations recapitulating mechanisms leading to lung fibrosis

Pulmonary fibrosis is one of the best characterized pathologies associated to CNTs exposures (Dong et al., 2015; Labib et al., 2016; Nikota et al., 2017; Sun et al., 2015). Although the development of pulmonary fibrosis is a complex process orchestrated by various cell types in the lung tissue, macrophages have a pivotal role in the initiation of the steps towards its development. While the complete set of MWCNTs-induced pathological alterations leading to fibrosis in the lung is still to be clarified, the early contribution of acute inflammation and ROS production has been already elucidated (Dong et al., 2015; Labib et al., 2016; Li et al., 2017). The increase of ROS production affects various cell types, and the inflammation promoting response in macrophages, specifically through NF-κB signaling, guides the biological system towards a fibrogenic response (He et al., 2011). Indeed, our results also highlight NF-κB activation (*cfr*. Paragraph 3.5 and Table S4). This type of signaling from macrophages is essential for the development of fibrosis, as the molecules secreted by macrophages regulate the function of other cell types, namely fibroblasts, in the tissue. In addition to NF-κB signaling, other signaling pathways are also relevant. For instance, TGF-b signaling, AKT/mTOR signaling, and WNT signaling, whose role in fibrosis has been extensively reviewed elsewhere (He and Dai, 2015), play a key role in the pathogenesis of fibrosis. All of these signaling pathways were found significantly enriched in our results (Table S4), and some of the key genes involved in these signaling pathways are represented in Fig. 5. Furthermore, recent evidence suggests that CNTs induce alternative macrophage activation both *in vivo* and *in vitro* (Dong and Ma, 2018; Meng et al., 2015; Kinaret et al., 2020). Mixed status of pro-inflammatory, M1-type and healing/regulatory M2-type macrophage activation has been associated to CNTs-induced fibrosis *in vivo* (Dong and Ma, 2018). Our observation on the induction of genes encoding for proinflammatory factors, such as IL-1β, CXCL-8, and TNF, suggest M1 activation, whereas the upregulation of profibrogenic mediators such as PDGFA, TGF-β2, VEGF-A, and CTGF together with anti-inflammatory IL-10, suggest the activation of M2-macrophages (Fig. 5, Table S2). The imbalanced combination of prolonged inflammation and persistent activation of M2-macrophages suggest pathogenesis of fibrosis (Dong and Ma, 2018; Braga et al., 2015). Interestingly, a further comparison of the DDD genes with known, rCNT-induced genes associated to lung fibrosis in an *in vivo* murine model, resulted in 55 common genes (out of 138) (Nikota et al., 2017). Altogether, these results suggest that our *in vitro* model of macrophage exposure is able to highlight relevant patterns of molecular alterations associated to the development of pulmonary fibrosis.

The involvement of DNA methylation in the progression of pulmonary fibrosis caused by MWCNTs has also been postulated (Brown et al., 2016). Indeed, we observed an early transcriptional induction and late promoter hypomethylation of the pulmonary fibrosis marker MMP-7 as well as the already mentioned CXCL-8, a chemokine associated with chronic inflammatory diseases and fibrosis in the lung (Rosas et al., 2008; Russo et al., 2014). These molecular changes suggest persistent expression of these genes through reduced gene promoter methylation. Our results also highlight DDD genes and gene promoters involved in events known to contribute to the development of fibrosis, such as cellular response to stress, alteration of calcium homeostasis, and protein metabolism in both molecular layers (Fig. 4, Table S4) (Ryan et al., 2014). Moreover, pathways found enriched only in the methylome were related to fibroblast growth factor receptor (FGFR) signaling (Table S4), further suggesting the role of DNA methylation in the macrophage response leading towards fibrosis (Inoue et al., 2002).

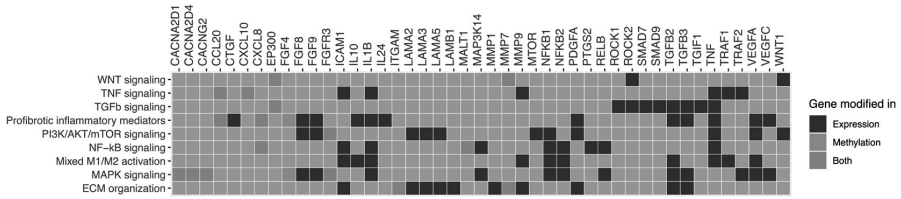

Fig. 5. Pathways and genes relevant to pulmonary fibrosis. Heatmap represents selected genes associated to signaling pathways and biological functions relevant for the pathogenesis of fibrosis. Colored squares denote the association of genes with biological functions. Blue indicates gene to have DDD alteration in expression, orange in methylation and turquoise in both, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

We have previously shown the potential of *in vitro* strategies coupled with advanced computational methods in informing on relevant outcomes of *in vivo* exposures (Kinaret et al., 2017a). In this study, we further utilized advanced toxicogenomics data modelling to investigate the complex mechanisms of adaptation in response to rCNTs exposure. We used PMA-differentiated THP-1 cells and a combination of multiple doses and time points. With this relatively simple model, we were able to highlight the activation of several well-known fibrosis-related genes and specific activation patterns suggesting potential long-term effects previously described *in vivo*. However, macrophages are a diverse group of cells, and differences between phenotypes and polarization status cannot be neglected (Kinaret et al., 2020). Regulatory bodies and the scientific community are currently putting significant effort to replace animal experiments with as short as possible *in vitro* testing with predictive power. This study brings valuable insights into obtaining evidence of potential long-term consequences from simple *in vitro* models when combined with robust and innovative computational strategies (Kinaret et al., 2017a). While our findings concerning the transcriptomic alterations are in line with multiple reports of CNTs effects on the gene expression, there is less data currently available concerning their ability to alter the DNA methylation. Moreover, the effects of ENMs exposures on other epigenetic mechanisms, including histone modifications, chromosome remodeling, and non-coding RNAs, are even less characterized (Gedda et al., 2019; Yu et al., 2020). Exploring the outcomes of ENMs exposures on these mechanisms can deepen the understanding of their toxicity. The approach used in the present study offers a valuable steppingstone for future integrated studies investigating other molecular alterations and the effects of different exposures.

While modelling the long-term effects from short-term *in vitro* exposures is not a simple task, our multi-omics approach to dynamic dose-dependent alterations is able to highlight macrophage responses both at the level of the transcriptome and methylome, and is able to suggest potential long-term effects already after a 72-h *in vitro* exposure set-up. These findings support the use of combined *in vitro* model systems and toxicogenomics approaches, simultaneously promoting the development of faster, cheaper, and more ethical testing strategies for ENMs.

4. Conclusions

Here, we report alteration of multiple genes and pathways with a key role in macrophage activation in response to rCNTs exposure. Our findings show distinct kinetics of adaptation in the transcriptome and promoter methylation. While macrophages respond at 24 h of exposure by mainly altering gene expression, as the exposure continues through 72 h, epigenetic mechanisms also have a role in macrophage adaptation. Our results convincingly suggest that our toxicogenomic approach of *in vitro* models informs on relevant pathogenic events observed *in vivo*.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.impact.2020.100274.

Declaration of Competing Interest

The authors declare no competing interests.

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PUBLICATION IV

A curated gene and biological system annotation of adverse outcome pathways related to human health

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A curated gene and biological system annotation of adverse outcome pathways related to human health

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Adverse outcome pathways (AOPs) are emerging as a central framework in modern toxicology and other fields in biomedicine. They serve as an extension of pathway-based concepts by depicting biological mechanisms as causally linked sequences of key events (KEs) from a molecular initiating event (MIE) to an adverse outcome. AOPs guide the use and development of new approach methodologies (NAMs) aimed at reducing animal experimentation. While AOPs model the systemic mechanisms at various levels of biological organisation, toxicogenomics provides the means to study the molecular mechanisms of chemical exposures. Systematic integration of these two concepts would improve the application of AOP-based knowledge while also supporting the interpretation of complex omics data. Hence, we established this link through rigorous curation of molecular annotations for the KEs of human relevant AOPs. We further expanded and consolidated the annotations of the biological context of KEs. These curated annotations pave the way to embed AOPs in molecular data interpretation, facilitating the emergence of new knowledge in biomedicine.

Background & Summary

Adverse outcome pathways (AOPs) are multi-scale models of biological mechanisms connecting molecular interactions between chemical exposures and biological systems (molecular initiating event, MIE) with adverse outcomes (AO) through key events (KE)[1]. KEs are measurable events described at increasing levels of biological complexity and connected through key event relationships (KER) that provide context and justification for the connection between the KEs. The AOP framework is central in modern toxicology, where efforts of shifting towards mechanistic models and alternatives to animal experimentation are taking place. AOPs can guide the development of new approach methodologies (NAMs) which include *in vitro* tests, targeted assays, and prioritisation strategies, and aim to fill the gaps in decision making in chemical risk assessment while also reducing the use of animal experimentation[2]. Similarly, AOPs can be applied to depict mechanisms of disease progression and other biological events[3,4]. AOPs not only provide a convenient framework to represent and interpret biology, but they also help to identify knowledge gaps and support the implementation of novel applications in biomedical research.

While AOPs model the cascade of events from a MIE to an AO at the level of tissues, organs, individuals or even populations, molecular mechanisms of chemical exposures can be investigated through toxicogenomics[5–7]. Toxicogenomics provides a complementary approach to the traditional observation of phenotypic effects of chemical exposures by focusing on the mechanism of action (MOA) of chemicals using omics technologies. This further enables an array of data-driven and computational approaches, including chemical grouping, read-across, and predictive models, and helps to explain why and how an exposure induces its effects[8]. This way, toxicogenomics can also inform the development of novel AOPs and support the application of AOP-based knowledge in the development of NAMs[9–14]. While to date the link between patterns of molecular alteration and

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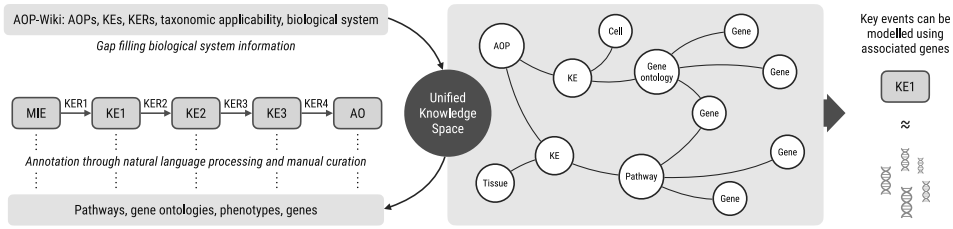

Fig. 1 Study overview. Data from the AOP-Wiki was embedded into a previously established knowledge graph, the Unified Knowledge Space (UKS)[24–26]. KEs of human relevant AOPs were annotated to pathways, gene ontology terms, phenotypes, and/or individual genes through natural language processing techniques and manual curation. Furthermore, the existing biological system (organ, tissue, cell type) annotations were amended. The knowledge graph structure was then used to associate genes mapped to the annotated terms to the KEs, allowing KEs to be modelled using sets of genes.

Data type	Resource	Link	Retrieval date	Version/Release
Pathways	KEGG[34]	https://www.genome.jp/kegg/pathway.html	10/14/2021	Release 100
	WikiPathways[33]	https://www.wikipathways.org/	10/14/2021	Version 20211010
	Reactome[35]	https://reactome.org/	10/9/2021	Version 78
Phenotypes	Human Phenotype Ontology[37]	https://hpo.jax.org/app/	10/14/2021	Release 2021-10-10
	KEGG disease[34]	https://www.genome.jp/kegg/disease/	10/14/2021	Release 100
Gene ontologies	Gene Ontology[36,40]	http://geneontology.org/	10/7/2021	Release 2021-09-01
Genes, gene products	Ensembl[41]	https://www.ensembl.org/index.html	10/31/2019	Release 98
AOPs	Aop-Wiki	https://aopwiki.org/aops.json	10/26/2022	Release 2.5
KEs, KE level, biological system	Aop-Wiki	https://aopwiki.org/events.json	10/26/2022	Release 2.5
KERs	Aop-Wiki	https://aopwiki.org/downloads/aop_ke_ker.tsv	10/26/2022	Release 2.5

Table 1. Data types and sources.

AOPs has been investigated at the level of individual or selected AOPs[13–16], a systematic framework to integrate these two concepts is missing. The primary challenge to this is the lack of thorough and robust annotation that would link biological events to meaningful sets of molecules (genes/proteins/etc.) whose alteration could be monitored through omics technologies. Establishing this link would enable straightforward interpretation of the complex patterns of molecular alteration in a mechanistic way.

AOP-related information is primarily stored in the AOP-Wiki repository (aopwiki.org). Varying levels of annotations (ontologies, taxonomic and life stage applicability, etc.) and metadata are provided to support the use of AOPs. The existing annotations, however, are only suitable to provide general context and associations between concepts, instead of allowing the modelling of the KEs through specific sets of genes. Furthermore, there are inconsistencies in the level of specificity and coverage of the annotations. Previous efforts of annotating KEs through computational approaches have been shown to be successful but they remained at the level of theoretical associations without the intention of modelling the KE-gene relationships[17,18].

Here, we present a comprehensive annotation of KEs relevant for human health to sets of genes. We integrated techniques of natural language processing (NLP) and manual curation to obtain robust and accurate associations. An initial version of this effort was used in a recent study to build AOP-based NAMs, including experimentally validated *in vitro* biomarkers for pulmonary fibrosis[19]. Furthermore, here we expanded the curation to fill gaps in the biological system annotations provided in the AOP-Wiki for the KEs. This helps to refine the AOPs and supports the reuse of existing KEs in new AOPs, which guides the identification of new links by enhancing the AOP network[14,19–23]. It can further improve applications combining AOPs with physiologically based pharmacokinetic (PBPK) modelling through the addition of relevant cell types, tissues, and organs. The overall strategy of the study is presented in Fig. 1.

Methods

Data structure and integration. The previously established knowledge graph, the Unified Knowledge Space (UKS)[19,24–26] was used as the foundation of the study. The knowledge graph is managed in Neo4j v. 4 (https://neo4j.com/), and the full list of data sources relevant for the present study is listed in Table 1 together with their data retrieval dates, versions, and references.

Pathways, gene ontology terms (GO), and phenotypes (together referred to as the *gene sets*) were introduced into the UKS as individual nodes with each term corresponding to a single node. Genes associated

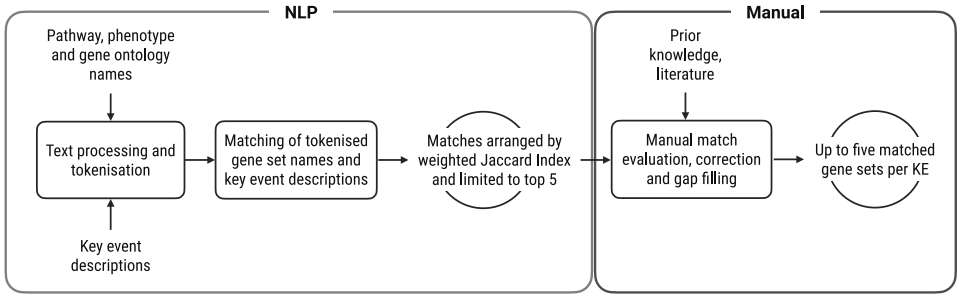

Fig. 2 Annotation strategy applied to link gene sets to KEs. Input to the pipeline is marked as text without an outline, process is outlined with a rectangular box, and output is marked by a circular outline. Orange outline indicates steps included in the natural language processing step, while blue outline marks manual curation.

with pathways and phenotypes were linked to them based on the data from each corresponding database, and the connections between gene ontologies (biological process, molecular function, cellular component) and genes were obtained from GO and Panther (Table 1). All genes were expressed in Ensembl gene identifiers for improved interoperability. AOP-related data were downloaded from the AOP-Wiki through the available API or through separate download files (Table 1) originally in November 2020 and updated in August 2022. AOPs were introduced into the UKS as individual nodes with connections to their associated KEs. Given the same KE can exist under multiple AOPs with distinct KERs, "Specific Key Event" (KE in the context of a specific AOP) nodes were added as descendant nodes of KEs. Labels such as "Molecular Initiating Event" and "Adverse Outcome" were assigned to the *Specific Key Event* nodes where applicable.

Annotation of key events to gene sets. KEs of AOPs relevant for human health risk assessment were annotated to gene sets through a multi-step procedure that combines NLP techniques with manual curation. The outline of the process is summarised in Fig. 2. An AOP was deemed relevant for human health if the reported taxonomic applicability included one or more of the following: *Vertebrata, Mammalia, Catarrhini, Rodentia, Homo sapiens, Rattus Norvegicus, Mus musculus*. The rodent species were included due to their important role as a model organism in human health risk assessment. AOPs with missing information of taxonomic applicability were manually evaluated based on the metadata provided in the corresponding AOP page and included if the pathway was biologically plausible for the selected organisms.

Initial matching and match prioritisation between KEs and pathways, phenotypes, and/or GO terms was performed using NLP techniques. The pipeline was established in Python version 3.7 using the packages nltk[27] version 3.6.7 and pandas[28,29] version 1.3.5. The KE descriptions and gene set names as expressed in the MSigDB[30] (collections H, C2 and C5) were converted to lower case and punctuations were removed. Further text processing included the replacement of concepts consisting of multiple words with one-word concepts using a custom dictionary m. For instance, word pair "positive regulation" was replaced by "upregulation". The preprocessed text was then split into tokens to be processed individually using the *word_tokenize* function. Tokens corresponding to common words that could lead to spurious matches (e.g., articles and prepositions), were detected and discarded using the list stop words provided by nltk. Finally, different declinations of the same concepts were mapped to the root terms using the *WordNetLemmatizer* available in nltk that makes use of WordNet's morphological modifications. This included the conversion of plurals into singular forms, different verb tenses into the basic form, as well as the standardisation of different spelling formats (e.g., "pparα" and "pparalpha" map both to "ppar-alpha"). As a result, each KE description and gene set name was presented as a set of tokens, e.g. {"upregulation", "ppar-alpha"}. Considering that the tokens appear in the KE descriptions and gene set names in varying frequencies, the informative value of each token is not equal. Rare tokens were considered more informative than the common tokens, hence, each token was weighted by its inverse document frequency (IDF)[31], $\mathrm{idf(t)} = \log(\mathrm{N/d})$, where N is the total number of gene sets considered and d is the number of gene sets that contain the token t. This means, that the weight of the token is inversely proportional to the number of gene set names and descriptions containing the token. These weights were then applied in the calculation of the weighted Jaccard Index (JIW)[32] between the sets of tokens x and y of each KE and gene set and used for the matching, $JI_w(x, y) = \frac{\sum_i \min(x_i, y_i)}{\sum_i \max(x_i, y_i)}$. Hence, rare tokens shared by KE descriptions and gene set names leads to a higher matching score than common tokens, making the results more specific. The matches were organised based on the JIW in descending order, and the top five matches were retained. Including up to five annotations for each KE allowed improved specificity and contextualisation when a single gene set would not result in a comprehensive match while also keeping the number of annotations manageable.

The prioritised matches were then manually evaluated and consolidated. This included the individual evaluation of all the matches for their accuracy and correct context, removal of irrelevant matches, and the refinement and gap filling. If the computationally prioritised matches were not biologically relevant or in the correct context, relevant gene sets were manually searched and added from the selected databases (WikiPathways[33], KEGG[34], Reactome[35], GO[36], Human Phenotype Ontology (HPO)[37]). At this stage, NLP-based matches derived from any

other database than the ones listed here were discarded due to limited representation. Given the goal of linking toxicogenomics data to the KEs/AOPs, the gene sets for KEs describing the alteration of an individual gene or gene product were linked to the main functions of the molecule (e.g., the activation of a specific gene or protein to the signalling pathway it drives instead of the individual gene), as such a signal is more likely to be captured from omics data than the specific induction of the gene (product) itself. However, if no distinct signalling pathways or key functions could be identified at the level of the gene sets, the KE was linked to the specific gene itself. If no biologically relevant matches could be identified, the KE remained unannotated. The hierarchical structure of the GO terms was exploited to add specificity to the gene sets by adding the relevant descendants for parent terms when applicable. For example, KE 1457 titled "Induction, Epithelial Mesenchymal Transition" was assigned the following GO terms: GO:0001837 - Epithelial to mesenchymal transition, GO:0010717 - Regulation of epithelial to mesenchymal transition, and GO:0010718 - Positive regulation of epithelial to mesenchymal transition.

The gene set names were mapped to the gene set identifiers and the results of the curation were integrated into the UKS as relationships between the KE nodes and gene set nodes. The level of the annotation (up to five annotations were provided to each KE) was included as an attribute of the edge, allowing future filtering based on the level. After establishing the links between KEs and gene sets, each KE can be represented as the union of all the genes associated to its matched pathways, GO terms and/or phenotypes. For this, human genes associated to each term were retrieved through the UKS.

Refinement of the biological system annotations. As part of the annotations in the AOP-Wiki, the level (molecular, cell, tissue, organ, individual, population) of the KEs is provided. Similarly, KEs are associated with a biological system that expresses the biological "location" of the KE. However, the provided locations may be limited to the context of the AOP in which the KE was first described. This can result in the duplication of KEs (i.e., the same event is added to the AOP-Wiki as a distinct KE resulting in the loss of the potential connection in a complete AOP network). Furthermore, this data is fully missing for some of the KEs. Completion of this information could improve network-based approaches to AOP research and the development of new AOPs. Hence, the existing biological system annotations were manually evaluated, refined, and extended to include plausible biological systems beyond the originally defined ones. Furthermore, gaps in the annotation were addressed and the already existing cell, tissue, and organ terms were amended with a system level annotation (e.g., respiratory system, endocrine system, etc.) and a cell component annotation, where applicable. For example, if a KE was annotated to a cell term "hepatocyte" in the AOP-Wiki, this annotation was supplemented with an organ/tissue term annotation "liver" and system terms "digestive system, exocrine system, endocrine system".

The annotations were assigned based on the primary location or context of the KE, as suggested in the AOP-Wiki. However, in cases where an organ or cell term was assigned in the AOP-Wiki, but other organs/tissues or cells were also determined as applicable, the original terms were replaced or amended with other possible organs, tissues, or cell types, with the options separated by "/". The KE descriptions (names) were used as the primary source of annotation, followed by the metadata provided for the KEs in AOP-Wiki. If the biological system was not clear based on the provided descriptions, a literature search was performed. For KEs at the molecular level (e.g., changes in the expression of individual genes/proteins), Human Protein Atlas[38] was used to determine relevant cell types and tissues. If the process was applicable for most or all cell types, "eukaryotic cell" was assigned as cell annotation, and the system and tissue/organ annotations were left unassigned to indicate the applicability of a range of tissues and organs. Furthermore, "eukaryotic cell" was introduced as a secondary annotation in cases, where the KE was specified for a distinct cell type or organ/tissue but would be biologically plausible in other cells, tissues, and systems as well. The secondary annotation was established to distinguish between any cells of a specific system, organ or tissue, and a generic eukaryotic cell. Finally, the systems, organs, tissues, cell types, and cell components were collected to a unified dictionary provided as part of the data collection.

Data Records

Data overview. The annotated data collection covers 231 AOPs with a total of 997 unique KEs that form 1636 AOP-KE pairs (specific KEs). Of these, 969 unique KEs (1,596 Specific Key Events) were successfully annotated to sets of genes. The number of gene sets associated to KEs ranges from 0 to 5 with a median of 3. Majority of the gene sets represent GO biological processes (total 1,532 annotations), followed by GO molecular function (273), Human Phenotype Ontology (263), Reactome pathways (195), WikiPathways (167), KEGG pathways (154), individual genes (89), and GO cellular components (83). The numbers in brackets correspond to total annotations of each type. The number of KEs at each level of biological organisation together with the proportions of annotation sources by KE level are shown in Fig. 3a. Total number of human-relevant terms present in each data source at the time of data retrieval (information available in Table 1) and their associated genes are summarised in Table 2.

Each KE is represented as the union of genes linked to its associated gene sets. For instance, Event:1493 "Increased Pro-inflammatory mediators" is represented as all the genes associated to its annotations "GO:0002532 – Production of molecular mediator involved in inflammatory response" and "GO:0006954 – Inflammatory response". Similarly, each AOP can be represented by the genes linked to its KEs. The number of genes in annotated KEs range from 1 to 6,047 with a median of 82, while the number of genes linked to the AOPs range between 15 and 6,381, (median of 804). In total, the annotations cover 16,825 genes with varying levels of specificity for KEs, i.e., some genes are associated with a large number of distinct KEs, while others are specific to individual KEs. This measure of KE specificity is an important factor in applications focused on the identification of KE specific biomarkers or reporter genes, for example. The distribution of the number of KEs per gene is presented in Fig. 3b.

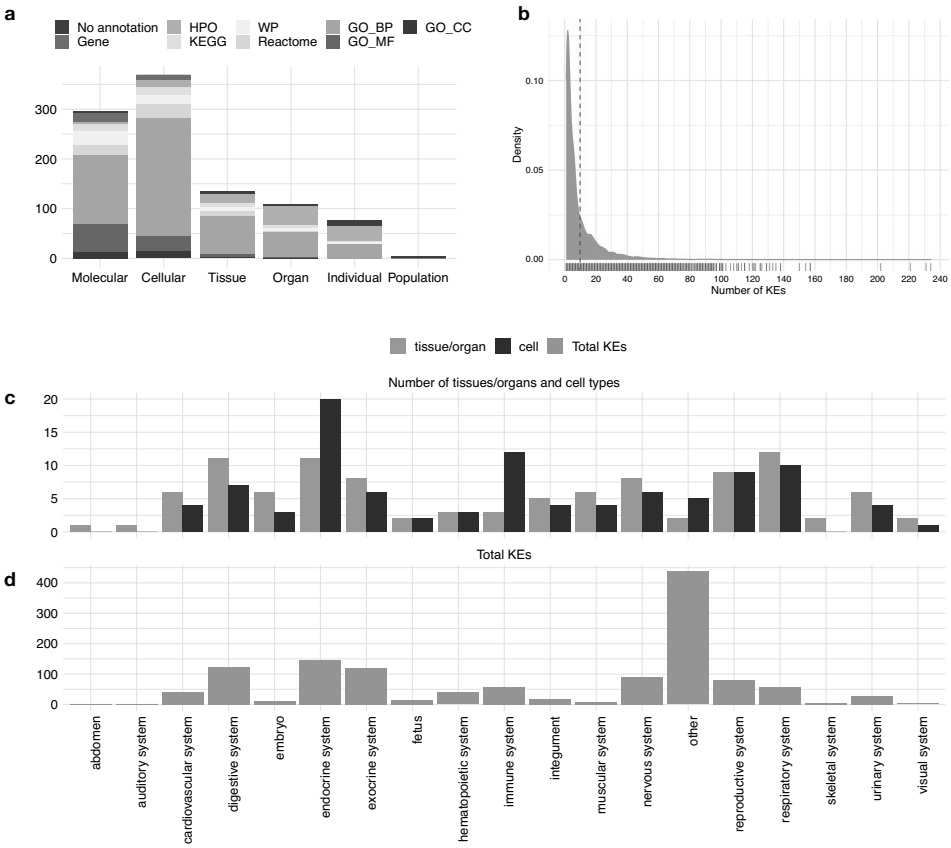

Fig. 3 Characteristics of the KE annotation. (**a**) Stacked bar plot representing the proportion of annotation types by KE level. Total height of the bar reflects the number of KEs in each level of biological organisation. Dark brown (no annotation) stack corresponds to the number of KEs with no associated gene sets, while the different annotation types are represented proportionally to their use in each level. HPO = Human Phenotype Ontology, WP = WikiPathways, GO_BP = Gene Ontology Biological Process, GO_CC = Gene Ontology Cellular Component, GO_MF = Gene Ontology Molecular Function. (**b**) The density distribution of the number of KEs associated with each gene. Median of the distribution is indicated with a dashed brown line and the rug below x-axis is used to support the interpretation of the distribution. (**c**) Number of different tissues/organs (turquoise) and cell types (dark green) under each system-level annotation. (**d**) Total number of KEs by system level annotation. The system "other" includes KEs assigned a cell type applicable for a range of tissues and/or systems, and those for which no system could be defined.

The biological system annotations were consolidated for all KEs available in the collection. In total, they cover 18 biological systems, 86 specific organs and tissues, and 70 cell types (Fig. 3c,d). Furthermore, 7 distinct cell components were defined. The annotations are provided at varying levels of biological complexity following the specification provided in the AOP-Wiki and the information in the KE description.

Data files and formats. The data[39] is available on Zenodo at https://doi.org/10.5281/zenodo.7980953. The provided files as summarised in Table 3.

Technical Validation

While the validity of the gene sets as models of KEs cannot be measures objectively at the scale of this study, we evaluated the consistency and robustness of the KE-gene set annotations by grouping together KEs with Jaccard similarity coefficient (JI) >0.90. In detail, JI was calculated between all pairs of KEs, and transformed into a distance matrix used for clustering. The clustering performed using the *hclust* function from R package stats with the complete method, and the optimal number of clusters was defined so that intra cluster JI was >90. As a

Data source	Terms included	Total terms	Genes included	Total genes
GO biological process	746	12380	8817	20411
GO molecular function	158	4434	5252	20878
HPO	171	9946	4233	5209
Reactome	108	2496	6894	12355
WikiPathways	69	701	3108	8808
KEGG	61	334	4097	9454
GO cellular component	49	1754	8434	21809

Table 2. Number of unique terms and genes used from each data source together with the total amount of human terms and genes present in each source at the time of data retrieval. The number of genes correspond to unique Ensembl gene identifiers.

Data type	Description	File structure	File name
KE to gene set annotation	KE to gene set annotations. Annotations provided by specific KE (AOP-KE pairs).	A spreadsheet file with two sheets, one with annotation provided as the gene set names, one with identifiers. Both sheets contain columns AOP, KE, Specific_KE, Description (KE name), and Match_1 through Match_5.	Gene_set_annotations.xlsx
KE to gene annotation	Direct KE to gene associations. KE associated genes are expressed as the union of all the genes mapped to the gene sets annotated to each KE.	File provided as a tab-separated text file. File contains two columns, one for the KEs and one for the genes. Genes expressed as Ensembl identifiers.	Genes_to_KEs.txt
Gene set identifier to name mapping	Mapping between gene set identifiers and the names used for matching KE descriptions to gene sets. File may be needed if genes are obtained from external sources.	File provided as a tab-separated text file. File contains two columns: term_name and exact_source.	Name_to_ID_mapping.txt
KE to biological system annotation	Annotation of KEs to relevant biological systems at the level of the system, organ/tissue, cell, and cell component.	A spreadsheet with a column for KE name, id, and level, as well as distinct column for each annotation by level, including the secondary annotations, and indication of duplication. Equal annotations are separated by "/".	Biological_system_annotations.xlsx
Dictionary	A complete listing of all the systems, tissues/organs, cell types, and cell components used in the biological context annotations.	A spreadsheet with five sheets. Complete dictionary covers all combinations of system, organ/tissue, and cell type annotations. Individual dictionaries provide a complete list of systems, organs/tissues, cell types, and cell components.	Dictionary.xlsx

Table 3. Description of files provided as part of the data set.

result, the KEs grouped into 731 clusters ranging in size from one to 19 KEs, with 128 clusters having at least two (Figure S1). We evaluated the clusters with six or more KEs (total six clusters). The largest cluster was characterised by KEs related to different types of cancer (19 KEs). These KEs were annotated to general pathways in cancer due to the lack of exact gene sets specific for the cancer type, while more specific annotations were available for KEs such as "Liver Cancer" (Event:1395) and "Breast Cancer" (Event:1193), leaving them outside the cluster. The second largest cluster (10 KEs) was formed by all cytotoxicity related KEs, followed by KEs regarding hormone release from the hypothalamus or the anterior pituitary gland (8 KEs), and inflammation (8 KEs). Inflammatory events are covered by a broad range of KEs ranging from different wordings of increased inflammation to more specific inflammatory events, such as "Increased Pro-inflammatory mediators" (Event:1491). The inflammation cluster was formed by the more generic processes, while the specialised KEs either formed a smaller cluster or stood alone. A fertility cluster (8 KEs) was formed by KEs describing decreased fertility and reduced reproductive success. Finally, cell proliferation formed a cluster of 6 KEs. A full list of the clusters is available in Supplementary Table 1 while the JI calculated between each pair of KEs is reported in Supplementary Table 2.

These results reflect the consistency and robustness of the annotations, while also highlighting the differences in ontologies and pathway curations for distinct biological processes. For instance, cancers like breast cancer and hepatocellular carcinoma are well covered and hence KEs of these processes could be assigned specialised gene sets, while liposarcoma and fibrosarcoma could only be matched with more generic pathways in cancer. As the curated pathways and gene ontologies evolve in specificity, the biological context annotations can provide a meaningful tool for refining the KE associated gene sets.

Usage Notes

The KE-to-gene set annotations presented in this manuscript result from an integrated approach, where computational prioritisation was performed using techniques of natural language processing, and further consolidated by manual curation to ensure appropriate context for the matches. Although this allows the human based assessment of each annotation, it is prone to potential interpretation errors and differences in views of priority and suitability of the matches. Here, the goal was to provide a comprehensive link between the AOP framework and omics data, hence the gene sets associated to KEs and AOPs should accurately reflect each process. While these gene sets do not replace the individual assays targeted for measuring individual KEs, they allow the identification of potential KEs and AOPs from complex molecular data, opening doors to various data-driven applications

to AOP development and use. Similarly, the biological context annotations are intended to support the reuse of KEs, and to guide the refinement of the AOP network and the discovery of hidden links between KEs. Although they were curated to accurately reflect the relevant biological locations for the KEs, they may not always include all possible options or exclude those that are not feasible. In practice, the annotations can be used to filter the data and/or the AOP network to only include KEs relevant to a biological system of interest or to merge redundant nodes. This may further result in connections between KEs that were previously not obvious.

These applications were supported by the indication of the gene set similarity as defined by the JI matrix provided (Supplementary Table 2). We previously observed several reasons behind identical gene sets between distinct KEs (JI = 1)[19]. Namely, these include 1) truly duplicated KEs; 2) the same event in different biological systems; 3) subsequent or related KEs mapped to the same terms due to inadequate specificity; and 4) opposite regulation of the same biological event (e.g., increased vs. decreased signaling), where the last case is also due to the lack of specificity in the available gene sets. We believe the consideration of duplicated KEs to be case-dependent. Certain applications may benefit from an approximate grouping based on the similarity of the associated gene sets (e.g., finding functionally related KEs), while others may rely on more robust and accurate refinement (e.g., merging nodes in an AOP network). While the users of these data are encouraged to find an approach that suits their application, the most robust set of duplicates based on semantics, gene set similarity, and the assigned biological context are identified and reported in Biological_system_annotations.xlsx file.

It is also worth noting that AOPs are under constant development, and individual entries are at different phases of completion. Only a handful of the AOPs available in the database are finalised and endorsed (aopwiki.org). This means that the majority of the AOPs and KEs included in this collection are subject to changes. Hence, we suggest the users to refer to the AOP-Wiki (aopwiki.org) for up-to-date information of KE relationships, KE-to-AOP mappings, and any further information that may support the use of this data.

All KE-gene annotations are provided as human gene sets. However, the selected taxonomies also include other species that are often used as model organisms in human health risk assessment. It is worth noting that some of the processes may not be directly applicable to humans. The exact species and the strength of evidence for taxonomic applicability for each AOP can be obtained from the AOP-Wiki. Additionally, the genes associated with the gene sets may differ from those reported in this study depending on the resource used to retrieve the genes. This may be due to the selected gene identifiers, updates in the original databases, as well as differences in the interpretation of hierarchical formats present in the databases (e.g., Gene Ontology). As an example, the GO gene sets used in this study are based on the direct annotations between GO terms and genes, while other resources may include genes annotated to all descendants of the term as well.

Code availability

Custom code and data used in the NLP-based prioritisation of the gene set annotations is available in the data repository[39] on Zenodo at https://doi.org/10.5281/zenodo.7980953 (file aop_mapping_nlp.tar.gz).

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Author contributions

L.A.S. Methodology, formal analysis, data curation, writing original draft. A.P. Methodology, data curation, writing original draft. S.K. Data curation, writing original draft. J.L. Data curation, writing original draft. A.S. Supervision, writing original draft. M.F. Methodology, software, writing original draft. D.G. Conceptualisation, methodology, funding acquisition, supervision, writing original draft.

Competing interests

The authors declare no competing interests.

Additional information

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PUBLICATION V

Toxicogenomics Data for Chemical Safety Assessment and Development of New Approach Methodologies: An Adverse Outcome Pathway-Based Approach

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Toxicogenomics Data for Chemical Safety Assessment and Development of New Approach Methodologies: An Adverse Outcome Pathway-Based Approach

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Mechanistic toxicology provides a powerful approach to inform on the safety of chemicals and the development of safe-by-design compounds. Although toxicogenomics supports mechanistic evaluation of chemical exposures, its implementation into the regulatory framework is hindered by uncertainties in the analysis and interpretation of such data. The use of mechanistic evidence through the adverse outcome pathway (AOP) concept is promoted for the development of new approach methodologies (NAMs) that can reduce animal experimentation. However, to unleash the full potential of AOPs and build confidence into toxicogenomics, robust associations between AOPs and patterns of molecular alteration need to be established. Systematic curation of molecular events to AOPs will create the much-needed link between toxicogenomics and systemic mechanisms depicted by the AOPs. This, in turn, will introduce novel ways of benefitting from the AOPs, including predictive models and targeted assays, while also reducing the need for multiple testing strategies. Hence, a multi-step strategy to annotate AOPs is developed, and the resulting associations are applied to successfully highlight relevant adverse outcomes for chemical exposures with strong in vitro and in vivo convergence, supporting chemical grouping and other data-driven approaches. Finally, a panel of AOP-derived in vitro biomarkers for pulmonary fibrosis (PF) is identified and experimentally validated.

1. Introduction

Mechanistic aspects of chemical exposures have been long exploited in the context of academic research, resulting in the emergence of toxicogenomics and systems toxicology as independent fields.[1,2] Although the mechanistic insight gained through the technologies employed in academia has been valued as supporting evidence in the regulatory setting, its incorporation into the regulatory framework is to date hindered by concerns related to the robustness and reproducibility of such data and its analysis.[3] At the same time, the growing need for faster, cheaper, and more ethical approaches for chemical safety assessment have made mechanistic toxicology central for clarifying aspects important to regulatory decision making. Furthermore, uncovering exposure related mechanistic properties is emerging as a fundamental approach for the design of new drugs and chemicals.[4,5] Hence, multiple high-end research initiatives are underway to drive the shift from traditional animal-based

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assessment of apical toxicity endpoints toward in vitro and in silico approaches supported by mechanistic evidence.[6–8]

Adverse outcome pathways (AOP) emerged as models to organize biological mechanisms into causally linked sequences of multi-scale events to support chemical risk assessment.[9] AOPs have since expanded beyond the limits of toxicology, showing their applicability in organizing mechanisms of disease progression and adverse health outcomes,[10,11] and could even be applied to assess beneficial effects of therapies. The mechanisms depicted by AOPs comprise a sequence of events that progress from the molecular initiating event (MIE) toward an adverse outcome (AO) through intermediate steps, key events (KEs), with biological complexity increasing as the AOP progresses. Individual KEs are connected by key event relationships (KER) that verbally explain the causal link between the events and provide context for the pathway.

The AOP concept quickly attracted attention due to its potential in tackling one of the major challenges in the shift away from traditional toxicology: deciphering systemic and long-term outcomes of chemical exposures without the use of animal experiments. While significant efforts still need to be made toward this goal, AOPs encompass the means to systematically guide the integration of in vitro-based evidence into the risk assessment framework.[12] AOPs provide the grounds for various predictive approaches, read-across, and the development of targeted assays and new approach methodologies (NAMs), as also suggested by regulatory agencies and international organizations, such as the OECD.[8] Furthermore, the construction of AOPs can help identify gaps in knowledge and guide resources toward mechanisms in need of further investigation, or alternatively, reveal connections that have not been previously characterized.[13]

Concurrently with the development of the AOP framework, the role of omics data in elucidating biomarkers and mechanisms of action (MOA) of chemical exposures and diseases has become more prominent.[14–18] Omics data have been used to support the development of AOPs, especially through the identification of molecular targets and mechanisms.[19–23] However, full exploitation of omics-based evidence in the context of AOPs is hindered by the complication of linking molecular data to complex biological events, affecting both the development and the application of AOPs. Furthermore, while the value of omics data in answering questions of regulatory importance is recognized, the complexity of its interpretation and the lack of standardization in analysis and reporting have hampered widespread regulatory acceptance of omics-based evidence.[24] Bypassing these challenges could broaden the application of AOPs, support the interpretation of complex omics data, and further aid in the development of the concept toward quantitative models and assays. While molecular assays based on arbitrarily selected reporter genes have been proposed (e.g., ToxCast assays), there is an urgent need to develop new data-driven unbiased molecular assays for reliable and efficient mechanistic safety assessment of chemicals.

Here we hypothesized that rigorous curation of molecular events associated with AOPs could facilitate the implementation of omics-based evidence to 1) guide the interpretation of omics data readout, 2) support the development of new AOPs, 3) identify and fill gaps in knowledge, and 4) transfer AOP-based knowledge into robust assays to support chemical safety assessment.

Well-curated gene ontologies, pathways, and biological processes are used to interpret omics results and their translation into biologically relevant information. While some KEs can be easily crosslinked with such terms and their associated genes, the annotation of complex KEs taking place at a higher level of biological organization (e.g., at the tissue- or organism-level) is a more demanding task. This requires knowledge regarding ontologies and the biological events themselves. For instance, generic annotations are helpful for categorizing KEs, but without the intention of modeling KEs using the associated gene sets, they will likely not reach the level of granularity required for such a task. This is currently reflected in the annotations provided in the AOP-Wiki repository (aopwiki.org). The annotation of KEs to selected ontologies is included as an option in AOP-Wiki. However, the coverage of the annotations is currently low and has not been intended for modeling the KEs using the gene sets associated with their annotations.

Previous efforts to curate external annotation have shown the potential of the approach.[25,26] However, these have either remained at the level of abstract associations or focused on individual examples.[27,28] Hence, systematic, fit-for-purpose, and up-to-date annotation linking KEs to curated gene sets has not yet been established. To this end, we applied an integrated strategy for defining gene-KE-AOP associations through systematic curation. We show the applicability of our strategy for evaluating potential AOs of chemical exposures, and for the identification of AOP-driven biomarkers that can inform the development of target assays and novel approaches to chemical hazard characterization.

2. Results and Discussion

We developed an integrated approach to systematically associate curated gene sets to the KEs and AOPs. Our approach combines natural language processing (NLP) techniques with manual curation to link relevant biological processes and pathways, as well as their associated genes, to KEs of AOPs relevant for human health risk assessment. The resulting gene-KE-AOP connections enable the modeling of KEs and AOPs through gene-level data, which further introduces novel ways to benefit from the AOP concept. We applied this approach to generate an AOP fingerprint for a known profibrotic exposure in vivo and in vitro and finally combined the annotation to a framework for prioritizing KE- and AOP-associated genes to guide the discovery of biomarkers and reporter genes. The complete approach described in the following sections is summarized in **Figure 1**.

2.1. The Majority of KEs can be Successfully Annotated to Curated Gene Sets

At the time of retrieving the data from the AOP-Wiki repository (November 2020), a total of 289 AOPs and 1131 distinct KEs were identified. However, after eliminating the AOPs for which taxonomic applicability was either not available nor in the scope of our investigation, 176 AOPs and 856 unique KEs remained, forming a total of 1245 unique AOP-KE pairs (specific KEs). Although the AOP-Wiki houses selected annotations for some of

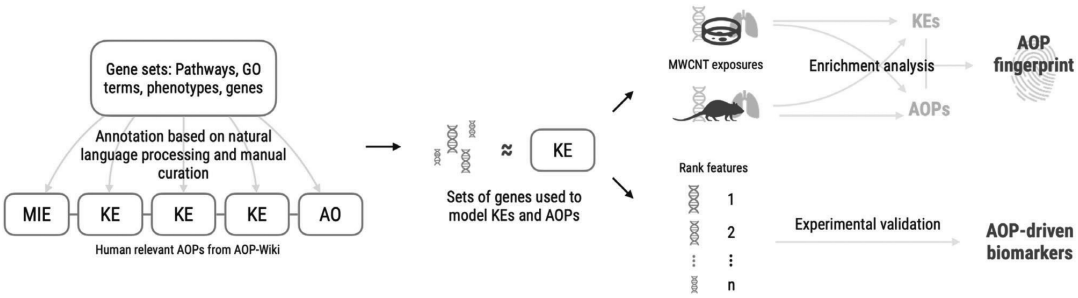

Figure 1. Overall scheme of the study. Established gene sets were annotated to KEs of the AOPs relevant for human health risk assessment. The resulting gene sets were then used to model the KEs. The validity of the annotation was evaluated using gene signatures of exposures with known adverse outcomes. Finally, we combined the approach with a gene prioritization framework resulting in the identification of AOP-driven biomarkers for pulmonary fibrosis.

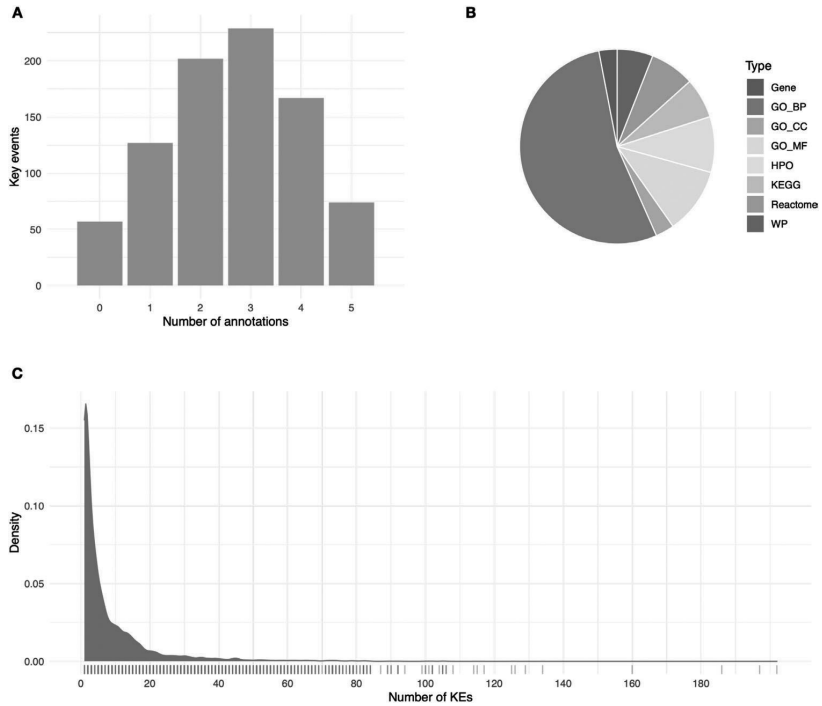

Figure 2. Descriptive analysis of the KE annotation. A) Bar plot describing the number of annotated terms per KEs. B) Pie chart expressing the proportions of different annotation types. C) Density distribution of the number of KEs each gene is annotated to.

the KEs, majority of them were considered not to be specific enough for our purpose (i.e., KEs describing the dysregulation of a specific gene annotated to terms such as "gene expression"). Additionally, as the existing annotations only cover a part of the KEs, we decided to consistently curate the annotation of all KEs. As a result, 799 unique KEs mapped to 175 AOPs received a curated annotation. The KEs were treated as individual instances, hence the same KE mapped to multiple AOPs was always annotated to the same term(s). A summary of the number of terms annotated to the KEs is presented in **Figure 2**A along with the proportions of the different term sources (Figure 2B). GO biological processes (GO_BP) represent most of the mapped annotations, followed by GO molecular functions (GO_MF) and human phenotype ontology (HPO). Since up to five annotations were provided for the KEs, the final gene sets used from herein comprise the union of the genes mapped to each annotated term. This structure

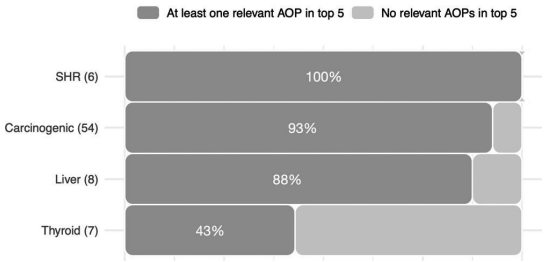

Figure 3. Bar plot representing the proportion of chemicals with relevant AOPs among the top five enriched AOPs based on the chemical classification. Number in brackets after the category name refers to the number chemicals in each category while the percentage on the bars reflects the proportion of chemicals in each category highlighting relevant AOPs. SHR stands for sex hormone receptor agonist.

allowed improved specificity, while also providing the possibility to further refine the gene sets using the hierarchical order implemented where applicable. The size of the gene sets associated to each KE range from one to 5990 genes, with a median value of 81 genes. Consequently, when AOPs are modeled by combining the gene sets associated to their KEs, the gene set sizes range from 15 to 5992 with the median size being 752 genes.

In total, the annotations comprise 15 825 genes. While the majority of genes are annotated to less than 5 KEs (9044 genes), 1434 genes have more than 20 KEs associated to them, and 50 genes have more than 80 associated KEs (Figure 2C). Although these numbers can be affected by annotation bias, for example, certain genes are better researched and annotated than others, they can also guide the selection of AOP-driven biomarkers when specificity is of importance.

2.2. AOP Enrichment Highlights Relevant Adverse Outcomes Associated to Chemicals

We tested the ability of our novel annotations to highlight relevant AOPs by analyzing a set of curated reference chemicals as defined by EU Reference Laboratory for alternatives to animal testing (ECVAM) and National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). We focused on four categories of chemicals defined by their toxicity properties to include hepatotoxic and carcinogenic agents as well as thyroid disruptors and sex hormone receptor (estrogen receptor—ER, and androgen receptor—AR) agonists. For each of the selected chemicals, we retrieved a list of associated genes from the Comparative Toxicogenomics Database (CTD),[29] resulting in a final set of 75 chemicals (File S1, Supporting Information).

First, we identified AOPs related to each of the selected categories (i.e., AOPs related to carcinogenesis, hepatotoxicity, sex hormones, and thyroid disruption) among all the AOPs. We then evaluated the prevalence of these relevant AOPs among the five most significantly enriched AOPs for each chemical. The results suggest that the enrichment approach successfully highlights AOPs of relevance for each group of chemicals (**Figure 3**). All sex hormone receptor agonists had at least one relevant AOP among the top five enriched, while the proportions varied from 43% (thyroid disrupters) to 93% (carcinogens) in the other categories (Figure 3).

In the group of carcinogenic chemicals, 93% of the compounds evaluated had cancer-related adverse outcomes among the top enriched AOPs. In fact, the group of carcinogens had the highest proportion of relevant AOPs at the top as compared to the others (median four out of five compared to the median of two out of five in the other groups). However, it should be noted that AOPs related to cancer are among the most represented group of AOPs, and cancer-related genes are generally highly researched and annotated, which may introduce a level of annotation bias that should be recognized.

The remaining four carcinogenic chemicals (7%) that showed no cancer AOPs among the top enriched AOPs were *N*-nitrosodiethanolamine, *N*-nitrosomorpholine, phenacetin, and tetrachloroethylene. *N*-nitrosomorpholine and *N*-nitrosodiethanolamine are both nitrosamines whose suspected AOs besides carcinogenesis include non-alcoholic steatohepatitis.[30] Indeed, both compounds contained hepatic steatosis related AOPs among the top five enriched AOPs (File S1, Supporting Information). Tetrachloroethylene (perchloroethylene, PCE) is a chlorocarbon solvent used in dry-cleaning and other degreasing applications.[31] AOPs with the most significant enrichment for PCE were also related to hepatic adverse outcomes. Although neurotoxicity is one of the most frequent AOs associated with PCE exposure, hepatotoxicity has also been reported.[31] Our results documenting liver steatosis are supported by biopsy-based evidence of liver disease, both in human and animal models, in settings of high occupational exposures.[32] Last, phenacetin is a drug that was widely used as pain medication until it was withdrawn from the market across the globe due to increasing evidence of carcinogenicity and renal toxicity.[33] The most enriched AOPs for phenacetin included immune related AOPs "Immune mediated hepatitis" (Aop:362) and Aop:277 titled "Inhibition of IL-1 binding to IL-1 receptor leading to increases susceptibility to infection". Although there is no described association between phenacetin and IL-1 or immuno-toxicity, it is known that they both play a role in paracetamol-associated liver toxicity, which is the main metabolite of phenacetin.[33,34]

In the case of the known liver toxicants, hexaconazole was the only compound not highlighting AOPs associated with liver toxicity among the top enriched AOPs. Hexaconazole is a widely used triazole fungicide. It acts by blocking sterol biosynthesis via inhibition of cytochrome P450.[35] Hexaconazole was considered as a Group C-Possible Human Carcinogen by the US EPA due to increased incidence of benign Leydig cell tumors in rats (https://www3.epa.gov/pesticides/chem_search/hhbp/R000356.pdf). Moreover, it was found to affect the reproduction of female rats.[35] The top enriched AOPs correctly identify this signature. Furthermore, the top two pathways "HMG-CoA reductase inhibition leading to decreased fertility" and "Modulation of adult Leydig cell function subsequent to decreased cholesterol synthesis or transport in the adult Leydig cell" both suggest a decrease in cholesterol levels by inhibition of the HMG-CoA reductase. Drugs inhibiting this enzyme, such as statins, are known to possibly cause liver damage.[36]

Known thyroid toxicants performed poorest in our analysis. Bifenthrin, malathion, permethrin, and simazine did not capture

thyroid related AOPs among the top five enriched. All these compounds have been widely used in agriculture as herbicides or pesticides. Agrochemicals represent a significant class of endocrine disrupting chemicals, albeit through varying mechanisms. It is now accepted that many of these molecules may mimic the interaction of endogenous hormones with nuclear receptors, such as estrogen, androgen, and thyroid hormone receptors.[37] Indeed, bifenthrin has already been reported as an endocrine-disrupting compound by blocking the binding of endogenous hormones.[38] In our framework, its anti-estrogenic activity emerges as the most enriched AOP (File S1, Supporting Information). Malathion is an organophosphate pesticide that is known for its low acute toxicity and rapid degradation.[39] In this light, it is not listed as a primary thyroid disrupting chemical, and its toxicity has been associated with the inhibition of acetylcholinesterase activity on nerve impulse.[39] Recent studies, however, demonstrated that malathion acts as an endocrine disruptor, both in vitro and in vivo.[40,41] Our results support these findings, highlighting the Aop:165: "Anti-estrogen activity leading to ovarian adenomas and granular cell tumors in the mouse" as well as Aop:112: "Increased dopaminergic activity leading to endometrial adenocarcinomas." Furthermore, Moore et al. demonstrated that malathion exposure at higher concentrations induces cytotoxic and genotoxic effects in HepG2 through oxidative stress, which can finally lead to liver cancer.[39] Similarly, our framework highlights both the "PPARalpha-dependent liver cancer" and "Cyp2E1 activation leading to liver cancer" AOPs. Simazine is a triazine herbicide whose use has been banned in most European countries for nearly two decades.[42] Simazine has now been recognized, similarly to the other compounds, as an endocrine disrupter.[42] Interestingly, the enrichment analysis for simazine highlighted AOPs related to the development of adenomas and carcinomas through endocrine disrupting activities (e.g., Aop:107 titled "Constitutive androstane receptor activation leading to hepatocellular adenomas and carcinomas in the mouse and the rat") as well as direct disruption of the GnRH pulse (File S1, Supporting Information). Although multiple in vivo and in silico evidence also indicate permethrin as possible endocrine disruptor,[43,44] no endocrine related pathways are present in the top enriched AOPs. However, this framework was able to highlight the modulating effect of permethrin on the lipid metabolism. It has been demonstrated that in HepG2 cells, permethrin increases lipogenesis and decreases beta oxidation, possibly contributing to the development of NAFLD.[45] Indeed, the "Inhibition of fatty acid beta oxidation leading to nonalcoholic steatohepatitis (NASH)" AOP is statistically enriched in our results.

Together, these results highlight relevant AOPs modeled by our curated gene sets to be enriched by the genes associated to the compounds, suggesting that our framework is able to support robust mechanistic and data-driven chemical grouping as well as the identification of potential AOs using chemical-gene associations.

2.3. Our Annotation Enables Grouping of KEs Resulting in Improved Modeling of the AOP Network

In order to fully unleash the potential of mechanistic toxicology, more informative testing strategies need to be developed that can monitor specific phases of the exposure-bio interactions and mechanisms. To this end, we defined accurate sets of genes capable of modeling specific KEs and AOPs. However, one of the challenges observed in the AOP-Wiki is the redundant semantics in the naming of KEs. While creating a new KE can be meaningful in many cases (e.g., the same biological process taking place in a distinct organ or a tissue), unnecessary redundancy can lead to challenges in the application of the AOP-based knowledge. This is especially true when modeling AOPs as a network and using such representation to identify hidden connections and to perform read-across analysis.[10,46–51]

Hence, we hypothesized that KEs could be grouped based on the degree of similarity of their associated gene lists. We calculated the similarity of the KEs based on their annotated gene sets and grouped together those mapped to identical sets of genes (Jaccard Index (JI) = 1). This resulted in the identification of 637 groups of varying sizes. These groups were characterized by four main concepts: 1) truly duplicated KEs due to distinct semantics; 2) same biological event in multiple biological systems; 3) subsequent KEs mapped to the same terms due to inadequate specificity; and 4) opposite regulation of the same biological event (i.e., increased vs decreased signaling).

Here, the grouping based on identical gene sets was selected due to the nature of the downstream application and statistical considerations (i.e., to avoid multiple testing against the same gene set in enrichment analysis). However, a parallel approach with varying cut-off values for similarity could be implemented to cluster KEs more roughly and to define specific categories of events. Similarly, further refinement of the KE clusters could help to enhance the AOP network by removing redundant nodes which, in turn, could reveal hidden links.

The potential of the KE grouping was showcased using a subgraph formed by considering the AOPs related to pulmonary fibrosis (PF). PF is a chronic lung disease characterized by tissue damage and scarring that impairs lung function.[52] A range of environmental exposures, including certain chemicals, drugs, radiation, and nanomaterials (most notably carbon nanotubes), as well as infectious diseases have been identified as causative agents for PF.[52–54] Moreover, the COVID-19 pandemic has raised concerns about increasing rates of PF.[55–57] Understanding the disease mechanisms can help in the development of strategies to treat and prevent the disease, and to control and modulate the exposures that contribute to its pathogenesis and progression. Furthermore, it can serve as the foundation for developing targeted assays for evaluating profibrotic potential of chemical exposures.

Six AOPs related to PF were available in the AOP-Wiki at the time of data retrieval (**Figure 4**A). These distinct AOPs characterize multiple pathways leading to the same AO. Together, these AOPs comprise 30 KEs, which form a connected graph when modeled as a network (Figure 4C). However, several redundancies were observed among the KEs. For instance, the AO was expressed either as *lung fibrosis* (Event:1276) or *pulmonary fibrosis* (Event:1458). Hence, the application of the similarity-based grouping resulted in 23 distinct KEs (Figure 4B) that were then used as the basis for merging the KE nodes in the PF network (Figure 4D).

The PF AOPs formed a connected network, indicating that each of the individual AOPs shared at least one KE with one or

A

AOP	AOP description
Aop:173	Substance interaction with the lung resident cell membrane components leading to lung fibrosis
Aop:206	Peroxisome proliferator-activated receptors γ inactivation leading to lung fibrosis
Aop:241	Latent Transforming Growth Factor beta1 activation leads to pulmonary fibrosis
Aop:319	ACE2 inhibition leading to lung fibrosis
Aop:347	Toll-like receptor 4 activation and peroxisome proliferator-activated receptor gamma activation leading to pulmonary fibrosis
Aop:382	Angiotensin II type 1 receptor (AT1R) agonism leading to lung fibrosis

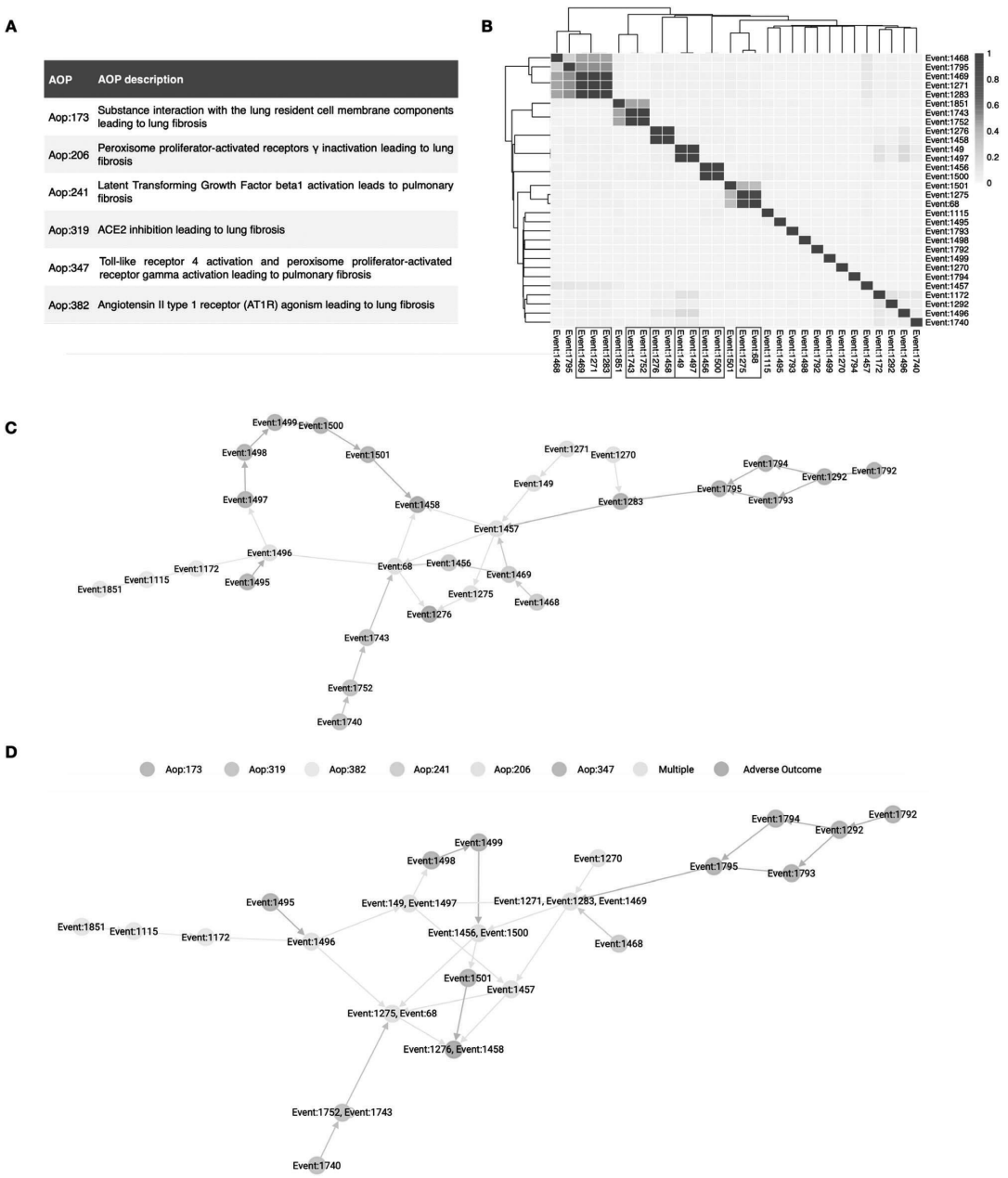

Figure 4. A) Table presentation of pulmonary fibrosis (PF) AOPs identified in the current study. B) Heatmap representing the Jaccard index-based similarity of the PF KEs as per their associated gene sets. Values close to zero (light gray) correspond to a low similarity between distinct KEs, while the increasing similarity is expressed with the color changing through green to blue. C) Graph presentation of the PF AOPs using their original KEs. Distinct colors denote the KEs of individual AOPs, gray nodes are KEs shared by multiple AOPs, and orange nodes correspond to the shared AOs. D) Graph presentation of the PF AOPs after KE grouping. The number of shared (gray) nodes has now increased, and the duplicated AO has been grouped into one distinct AO (orange).

Figure 5. AOP fingerprint of Mitsui-7 exposure in vitro and in vivo. Size of the bubble reflects the proportion of significantly enriched KEs in an AOP, while to color denotes the FDR-adjusted *p*-value in a negative logarithmic scale (i.e., the higher the number, the smaller the *p*-value). The AOPs are organized by the enrichment *p*-value from the in vivo data.

more of the other AOPs. However, as the duplicated KEs were merged, the similarities between the AOPs became more evident. This is evidenced by the increasing number of shared KEs in the graph after merging (the gray nodes in Figure 4D) as compared to the original graph (Figure 4C). Furthermore, the merging revealed Aop:206 to be fully contained within the other AOPs.

The refinement of the AOP network through KE grouping simplifies the network while also enhancing the robustness of the KE relationships, depicted by the connections between the nodes. This process, in fact, removes redundant nodes, which supports the application of AOP networks in AOP research and risk assessment. Furthermore, as duplicated events are removed, the true influence of each node can be assessed more robustly through network analytics.

This example demonstrates the effect of KE redundancy and the potential of data-driven grouping of the KEs. While manual assessment and grouping would be achievable for a limited number of AOPs at a time, doing it AOP-Wiki wide would be a massive undertaking. Here, we show how our curated gene-KE-AOP connections can help guide the grouping and hence enhance network-based approaches in AOP research. Furthermore, our results suggest that it is often possible to identify genes that can successfully represent multiple similar key events.

2.4. The AOP Fingerprint of Multi-Walled Carbon Nanotubes Converges In Vitro and In Vivo

Toxicogenomics has supported the development of mechanistic toxicology and further enhanced the possibility to obtain relevant information from in vitro studies, which could reduce the need for animal experimentation.[58–60] Here, we tested the hypothesis that toxicogenomic data generated in two independent in vitro and in vivo exposure models would converge on a robust set of relevant AOPs. We focused on Mitsui-7, a known hazardous long and rigid multi-walled carbon nanotube (MWCNT). The airways provide the most prominent route of exposure to this nanomaterial, and it is best characterized for its lung-related AOs, including PF.[61–64] Hence, we selected data derived from a lung exposure to the MWCNT in mice,[65] and an in vitro dataset with exposures on four cell lines representative of the human airways.[59,66] These cell lines include differentiated THP-1 cells as a model of macrophages, A549 representing alveolar basal epithelial cells, BEAS-2B as bronchial epithelial cells, and MRC9 as a model of lung fibroblasts. Differentially expressed genes (DEGs) from all experimental conditions in vivo and each cell line in vitro were obtained from Saarimäki et al.[67] and merged into a single MOA in vivo and in vitro, respectively.

We then performed enrichment analysis against both the AOPs and the KEs separately in order to evaluate the coverage of distinct KEs. We used the proportion of significantly enriched KEs to further filter the significant AOPs. This led us to identify 33 significant AOPs from the in vivo data, while 12 resulted significant from the in vitro exposure. These results were defined as the specific AOP fingerprint for the exposures, and it is presented in **Figure 5**.

Despite the distinct sizes of the AOP fingerprints, ten of the 12 AOPs enriched in vitro were also included in the in vivo fingerprint. Moreover, the top enriched AOPs were shared and ranked similarly between in vivo and in vitro when ranked by the

smallest adjusted *p*-value. The AOP enriched with the most significant *p*-value in both instances was Aop:173 titled "Substance interaction with the lung resident cell membrane components leading to lung fibrosis" (Figure 5). The in vivo data set was able to capture seven of the eight KEs of the AOP as significantly enriched, while three out of the eight KEs were enriched in vitro. Interestingly, Aop:173 has been specifically developed with robust evidence from MWCNT exposures, and multiple types of carbon nanotubes are listed as known stressors for the AOP (https://aopwiki.org/aops/173). The second AOP (Aop:171), on the other hand, describes the induction of pleural/peritoneal mesotheliomas by chronic cytotoxicity in rats. Like most AOPs used in this study, Aop:171 is still under development and lacks information on potential stressors. However, mesothelioma is a well-known AO of asbestos exposure, a fibrous silicate mineral whose adverse effects have often been used as a warning example for MWCNTs.[68] Indeed, similarities in their MOA have been extensively investigated.[61,69,70]

The in vitro AOP fingerprint captures effects such as frustrated phagocytosis, oxidative stress, cytotoxicity, and immune activation, which have all been reported as consequences of this type of exposure and contribute to the pathogenic nature of Mitsui-7.[61,62,64,71] Similarly, the profibrotic effects are highlighted with the multiple PF AOPs enriched. These effects are also observed in the in vivo AOP fingerprint. However, the in vivo fingerprint further highlights various AOPs outside the respiratory system, which is less apparent in vitro. While AOs beyond the immediate exposure site are feasible, many of these could likely be accounted for by the different effects of similar transcriptomic signatures in different biological systems (e.g., multiple AOPs related to gastric ulcer formation could reflect similar mechanisms of surfactant disturbance in two distinct exposure sites). On the other hand, the AOPs unique to the in vitro fingerprint, Aop:277 and Aop:263 (Figure 5), reflect the specific effects of the Mitsui-7 exposure on the immune system. Such specific signals can be easily masked in the in vivo system, where a large array of cell types is affected by the exposure.

It is worth noting that the exposures selected for the analysis had diverse set ups and a notable difference in the size of the combined MOA (863 DEGs in vitro versus 3540 in vivo). While data from multiple cell lines were selected to capture effects besides immune cell activation in vitro, we were not able to match the dose and time course set up present in the in vivo dataset. However, we wanted to include this long-term exposure to evaluate whether it would result in broader coverage over the KEs of AOPs. Furthermore, histopathological evaluation from the same in vivo exposure set up has shown fibrosis in the lung from the day 7 onward,[72] suggesting that a whole PF AOP could be covered with this data. Indeed, all but the MIE (Event:1495) of Aop:173 were enriched in vivo. The high proportion of enriched KEs in the in vivo data supports the modeling of KEs with relevant gene sets and the use of toxicogenomic evidence for the development of AOPs, as well as the evaluation of potential AOs of chemical exposures. Likewise, we show that the analysis of toxicogenomic data against robustly annotated AOP framework supports a high degree of in vitro to in vivo extrapolation and further supports the inclusion of toxicogenomics-based evidence for regulatory purposes. The concept of the AOP fingerprint can be easily adapted to evaluate other chemical exposures and AOs. With nearly 16 000 genes mapped to the KEs in our curation, they are expected to cover most of the human genome. Hence, the AOP fingerprint provides a robust framework for meaningful interpretations also for chemicals and phenotypes that may be less characterized.

2.5. KE-Associated Gene Sets Guide the Selection of Biomarkers

We showed that our KE-linked gene sets provide a robust way of evaluating potential outcomes of chemical exposures from transcriptomics data. This observation alone can help to guide chemical testing and grouping. However, to support the development of target assays and integrated approaches, specific reporter genes and markers need to be identified.

Selection of transcriptional biomarkers and reporter genes only based on differential expression from experimental data gives little context or reference to the AO of interest. Even if a certain exposure is known to induce a specific endpoint, there is no indication whether the measured deregulation could be associated with the phenotype of interest. On the other hand, prioritizing the features in the context of the KEs or whole AOPs could shed light on the importance and specificity of the feature regarding the phenotype. This, in turn, can guide the selection of potential biomarkers even in the absence of experimental data. Hence, we implemented a universal and customizable framework for the prioritization of the KE-associated genes to drive the identification of AOP-informed biomarkers and used it to identify AOP-driven biomarkers for PF. The shortlisted marker genes were then screened by RT-qPCR in an in vitro model of human macrophages exposed to bleomycin, a well-known profibrotic chemical.[73]

First, we defined characteristics for optimal biomarkers based on the Bradford Hill criteria, originally defined to evaluate causality in epidemiological research,[74] but later adopted to other research fields as well.[75] Our newly defined characteristics, their Bradford Hill counterparts, and short descriptions of the consideration of each step in the selection process are summarized in **Table 1**.

The prioritization and selection of the candidate biomarkers considered three main aspects: 1) the social life of genes, that is, some genes (gene products) are more influential than others; 2) specificity regarding the endpoint of interest; and 3) experimental evidence suggesting the genes respond to a relevant exposure. The ranking of the genes was based on the first two considerations, while the experimental evidence was included to guide the selection of candidate genes for RT-qPCR validation from the ranked list. This enabled a flexible selection process that would be applicable even in the absence of experimental data. At this stage, we also considered the biological feasibility of the target genes given the selected macrophage model as well as a broad coverage over the PF KEs.

As a result, we obtained a list of 25 candidates out of the original 2075 genes related to PF (**Table 2**). Although we focused on the genes in the top 10%, we further included genes ranking lower to obtain a broader coverage over the PF KEs. Genes that are specific to individual KEs might rank low when the individual lists are combined. Hence, we considered the expression patterns from the experimental data as well as the specificity scores

Table 1. We defined characteristics for optimal biomarkers based on the Bradford Hill criteria. The characteristics were then implemented into the prioritization and selection protocol, and further to the evaluation of the prioritized genes.

Bradford Hill	Our characteristic	Method/Assessment
Consistency (reproducibility)	Reproducibility	Selection considers evidence from previous profibrotic exposures
Strength (effect size)	Amplitude	Significant alteration of the expression as compared to control
Experiment	Measurable	Transcriptional biomarkers measurable by qPCR; selected genes need to be expressed in the model
Biological gradient (dose-response relationship)	Dose-responsive	Benchmark dose modeling to evaluate dose-response
Coherence	In vitro to in vivo extrapolation	Experimental evidence from in vitro and in vivo[a)]
Analogy	Predictive (of the outcome of interest)	Selection based on the KEs preceding the AO of interest
Specificity	Specificity	Gene ranking based on the specificity score
Plausibility	(Biological) plausibility	The AOP framework provides a plausible context; supporting evidence; selection of the organism
Temporality	Temporality	Transcriptional alteration follows the exposure; selection of the model organism[b)]
–	GLP-method	RT-qPCR
–	Influence	Centrality measures from human protein-protein interaction and gene regulatory networks

[a)] The biomarkers selected here are targeted for the development of non-animal assays for toxicological assessment. Hence the coherence to in vivo set ups is not evaluated experimentally. However, in vivo data was used for the selection of the markers to provide context of the systemic response [b)] Temporality in the Bradford Hill criteria refers to a clear distinction of the exposure happening prior to the outcome. Here, we considered temporality by observing transcriptional changes post exposure as well as in the selection of the model organism. Macrophages have a crucial role in the initiation of the profibrotic response preceding the outcome, fibrosis.

and ranks in the individual KEs. This also allowed us to evaluate whether genes ranked higher would perform better than others.

We could detect the expression of 22 of the candidate genes at one or more of the evaluated time points, and six of the detected genes showed significant alteration as compared to the unexposed control samples (Table 2). Finally, five of these genes were altered in a dose-dependent manner: CXCL2 and CCL7 at 24 h, IL8 (CXCL8) at 24 and 72 h, and MMP19 and TGFBI at 72 h. All but TGFBI of these genes were among the top 10% in the global PF rank (Table 2). Although we were not able to fit a dose-dependent model on the highest ranked gene, SMAD7, a suggestive trend could be appreciated in its expression pattern (Figure S4, Supporting Information, panel 18/6H). The expression of each of these genes was upregulated as compared to the controls (Figure S4, Supporting Information).

The central role of TGF-beta signaling is well-established in PF,[76] but neither of the TGFB genes tested (TGFB1 and TGFB3) showed significant change in expression in our setup. Indeed, TGF-beta is activated through a complex cascade of events, where the inactive form of the protein is activated by other effectors post-translationally,[77] making members of the TGF-beta family a more attractive target for protein-based biomarker assessment over gene expression. At the same time, we did observe upregulation of SMAD7 and TGFBI which are both activated by TGF-beta,[78,79] suggesting the induction of TGF-beta signaling. The protein encoded by TGFBI is involved in the extracellular matrix (ECM), and it has been shown to bind type I collagen, resulting in thicker fibers and further affecting macrophage polarization toward the M2 type.[80] Indeed, bleomycin has been suggested to polarize macrophages toward M2 (often referred to as the anti-inflammatory type), which have been shown to drive the development of PF through their ability to promote myofibroblast differentiation.[81,82] Many of our suggested biomarkers are chemokines that mediate immune responses. IL8 and CXCL2 are best characterized as neutrophil attractants, while CCL7 targets a wide variety of leukocytes.[83–85] Indeed, prolonged inflammation, combined with persistent M2 macrophage activation, supports pathogenesis of fibrosis,[86] and a mixed status of M1/M2 macrophage activation has been previously associated with carbon nanotube-induced PF in vivo.[87] Similarly, multi-walled carbon nanotubes have been shown to induce the polarization of macrophages toward such mixed status of M1/M2 polarization.[88,89] MMP19 is a member of the matrix metalloproteinase family involved in ECM remodeling.[90] MMPs have been extensively characterized in the context of PF,[91,92] and MMP19 specifically has been suggested as a key regulator of PF in mice and humans.[93]

Although macrophages alone cannot capture all the KEs of PF, our model is able to highlight the key steps of macrophage involvement in PF. The temporality of the expression of our suggested biomarkers is supportive of the events leading to the development of fibrosis, where the initial inflammation is followed by type M2 macrophage activation that together contribute to the development of a profibrotic microenvironment and responses in other cells in the tissue.[86]

NAMs are urgently needed to reduce animal testing while providing robust evidence to support chemical safety assessment. Although alternative methods have been successfully developed to capture acute effects, modeling long-term outcomes of the exposures, such as fibrosis, in vitro is still a challenge. Here, we propose a panel of five genes CXCL2, CCL7, IL8, MMP19, and TGFBI as AOP-derived robust biomarkers of PF to be

Table 2. Genes selected for qPCR validation. Green = yes, white = no.

Gene (rank)	Time point	Detected (Amplification)	Deregulated (ANOVA)	Dose-dependent
	6 h			
SMAD7 (1)	**24 h**			
	72 h			
	6 h			
CXCL2 (3)	**24 h**			
	72 h			
	6 h			
SPP1 (18)	**24 h**			
	72 h			
	6 h			
CCL2 (19)	**24 h**			
	72 h			
	6 h			
TGFB1 (23)	**24 h**			
	72 h			
	6 h			
IL8 (33)	**24 h**			
	72 h			
	6 h			
LOX (48)	**24 h**			
	72 h			
	6 h			
PLOD2 (74)	**24 h**			
	72 h			
	6 h			
MMP7 (80)	**24 h**			
	72 h			
	6 h			
CXCL10 (91)	**24 h**			
	72 h			
	6 h			
CCL7 (93)	**24 h**			
	72 h			
	6 h			
MMP9 (105)	**24 h**			
	72 h			
	6 h			
LTBP4 (112)	**24 h**			
	72 h			
	6 h			
FN1 (116)	**24 h**			
	72 h			
	6 h			
GDF15 (153)	**24 h**			
	72 h			
	6 h			
MMP19 (179)	**24 h**			
	72 h			

(*Continued*)

Table 2. (Continued).

Gene (rank)	Time point	Detected (Amplification)	Deregulated (ANOVA)	Dose-dependent
	6 h			
PTX3 (220)	**24 h**			
	72 h			
	6 h			
TGFB3 (297)	**24 h**			
	72 h			
	6 h			
LTBP3 (335)	**24 h**			
	72 h			
	6 h			
TWIST1 (609)	**24 h**			
	72 h			
	6 h			
TGFBI (727)	**24 h**			
	72 h			
	6 h			
CTSK (759)	**24 h**			
	72 h			
	6 h			
RCN3 (1592)	**24 h**			
	72 h			
	6 h			
RSAD2 (1596)	**24 h**			
	72 h			
	6 h			
PLK3 (2027)	**24 h**			
	72 h			

successfully measured in a model of human macrophages in vitro after short exposure time.

3. Conclusion

Mechanistic toxicology encompasses the means for faster, cheaper, and more ethical chemical safety assessment. However, to unleash the full potential of mechanistic evidence also in the regulatory framework, robust approaches to build confidence toward toxicogenomics are urgently needed. Here, we presented an integrated approach that links toxicogenomics with the concept of AOPs and proved its applicability to chemical grouping and development of data-driven NAMs. We introduced the AOP fingerprint, a concept for evaluating potential systemic outcomes of chemical exposures through unbiased interpretation of toxicogenomics data. Our analysis points to a consistent AOP fingerprint of MWCNTs extrapolated from both in vitro and in vivo experiments. Finally, we identified and experimentally validated a panel of robust AOP-derived in vitro biomarkers for PF.

Our results suggest that combining the regulatory-supported AOP framework with toxicogenomics through a rigorous mapping of the MOA of chemicals into KEs/AOPs can facilitate the inclusion of omics derived evidence in regulatory evaluations. The outcome of our analysis in the form of the AOP fingerprint provides a clear and easily understandable way to summarize complex omics data while providing robust statistical evaluation that can support regulatory decisions. Moreover, the possibility to use the framework suggested in this manuscript as the foundation for developing data-driven molecular assays further opens new possibilities for faster regulatory acceptance of novel alternative methods and NAMs.

4. Experimental Section

Definition of Knowledge Graph-Based Data Structure: A knowledge graph-based data structure was established by expanding the previously introduced framework, the Unified Knowledge Space (UKS) by the authors.[94,95] A detailed description and a full list of integrated data sources are provided in the Supporting Information. The so formed data structure was utilized throughout the study as described in the following sections.

Annotation of Key Events: A multi-step strategy comprising NLP and manual curation was applied to annotate KEs to established gene sets through pathways, phenotypes, and gene ontologies. The annotation strategy is summarized in Figure S2, Supporting Information.

Computational prioritization of KE annotations: To match the descriptions of key events and gene sets an NLP pipeline (Figure S2, Supporting Information) was developed. The pipeline performed several operations to extract the informative terms from both the descriptions of a key event and a gene set that were scored to assess the degree of matching between the two entities. In detail, first, the raw text was converted to lower case and all punctuation symbols were removed. Second, concepts that span multiple words in the text description were replaced by a single word expressing the same concept to strengthen the matching quality (e.g., the concept "positive regulation" was replaced with the single word "upregulated"). Third, the text was split into tokens which were further processed one by one. Fourth, each token corresponding to a stop word in the English language was dropped. Stop words refer to the most common words in a language that do not bring additional meaning (e.g., for the English language common stop words include "in", "the", "of", "from"). Fifth, different declinations of the same concept were mapped to their root term (e.g., plurals were converted to singulars, the terms "increased" and "increasing" were both mapped to "increase"). This same procedure was also used to standardize several styles to write the same symbol (e.g., "pparα" and "ppar-alpha" map both to "ppar-alpha"). After these preprocessing steps, each gene set and key event was represented by a set of token words, for example, {upregulate, ppar-alpha}. However, the frequency of each token word across the descriptions of genes and key events was not the same, and hence, the informative value of rare terms was higher than the informative value of more common tokens. This was taken into account by weighting each token by its inverse document frequency (IDF), that is, the weight was inversely proportional to the number of gene sets and key events that contain that token. Finally, a weighted version of the Jaccard Index (JIW) was employed to match gene sets and the key events, using the IDF as weights (i.e., each token that was shared between a gene and a key event did not account for 1 as in the standard JI, but it contributed its IDF weight to the matching score) and the matching gene sets for each key event was sorted in descending order.

Manual curation and refinement of annotations: Next, the results of the computational prioritization were manually evaluated for correct context and accuracy. Manual curation was used for gap filling and refinement of the annotations. In detail, the top five matches retained from the NLP-based approach were evaluated, and inaccurate or spurious matches were discarded. In case no matches from the computational prioritization were deemed suitable, a manual search related to the name of the KE was performed on relevant databases (WikiPathways,[96] HPO,[97] KEGG,[98] Reactome,[99] and GO [100]). For molecular level KEs, where the alteration of an individual gene was described, either the main functions of the gene were selected, or the gene was directly annotated to the ensemble identifier of the said gene. More generic annotations (i.e., annotation of a KE describing the alteration of a gene to a functional term tightly related to that gene) were prioritized to increase the size of the relevant gene sets.

The matches for KEs expressing the increase/activation or decrease/repression of a biological progress were further organized based on the hierarchy of the terms by prioritizing the most generic but suitable term followed by increasing specificity when multiple annotations of various specificities were available. For instance, Ke:1457 called "induction, epithelial mesenchymal transition" was annotated to the following terms: 1) epithelial to mesenchymal transition (GO:0 001837); 2) regulation of epithelial to mesenchymal transition (GO:00 10717); and 3) positive regulation of epithelial to mesenchymal transition (GO:00 10718). The curated KE-gene set links were added to the UKS so that for each key event entity its top five matches were added, while the matching level was stored as an edge attribute. This allowed to either combine multiple mappings for a key event or to filter for specific mapping levels. Since the KE-gene set mappings were always the same for the same KE, these relationships were added to the *Key Event* entities and not to the *Specific Key Event* entities, which reduced complexity of the knowledge graph as well as reduced needed storage space. The information, however, could still be retrieved from the UKS via its connecting paths.

Gene set retrieval: The genes corresponding to the matched terms were retrieved by matching the term names to their exact identifiers and querying the UKS for human genes associated with the terms. For phenotypes (HPO and KEGG disease), only genes with a link in the original database were included by filtering by the source for the connection. In cases where no human genes were linked to the annotated GO term, the mouse and rat genes associated and converted them to human orthologs using Ensembl,[101] which were then used as the corresponding gene sets. When no genes of human, mouse, or rat were associated with the original term, the annotation match was discarded and considered unsuccessful. Once gene sets to all original terms were defined, the gene sets were merged to obtain the final set of genes corresponding to each KE in this study.

Enrichment Analysis of Reference Chemical-Associated Gene Sets: To evaluate the ability of this framework to highlight relevant adverse outcomes from chemical associated gene signatures, lists of reference chemicals were retrieved from the ECVAM reference chemical library[102] and the NICEATM website (https://ntp.niehs.nih.gov/whatwestudy/niceatm/resources-for-test-method-developers/refchem/index.html). From the resources provided by ECVAM, a hepatotoxic chemical list that had clear distinctions between positive and negative compounds was selected. This list was based on the work from EPA's Virtual Liver project (https://cfpub.epa.gov/si/si_public_record_report.cfm?dirEntryId=166616&Lab=NCCT), and was provided as a downloadable Excel-file (./CHELIST/CheLIST__EPA_VLIVER.xlsx) by ECVAM. From NICEATM, the list of chemicals with characterized thyroid activity (specified as "ACTIVE" in the listing produced based on a previous publication by Wegner et al.) was selected.[103] AR and ER agonists were selected from the lists of in vitro reference chemicals provided on the website. These listings had been previously published in Kleinstreuer et al.[104] and Browne et al.,[105] respectively. Finally, carcinogenic compounds were identified from the list containing chemicals that were either known carcinogens or reasonably anticipated to be human carcinogens (RAHC) based on the 14th report on Carcinogens (RoC classifications) provided by NICEATM. The chemicals from each of the reference lists were then matched to the list of chemicals obtained from the CTD[29] through name-based matching or by the provided CAS identifiers, resulting in the final lists of reference chemicals for each endpoint used in this study. Chemical-gene links originating from the CTD were retrieved from the UKS and only chemicals with 50–1000 associated genes were included in the enrichment analysis. This filter was applied to minimize the false discovery rate and spurious matches in the enrichment analysis.

Enrichment analysis was performed using the Fisher's exact test as implemented in the function *enrich* from R package bc3net[106] for each chemical associated gene set against the list of AOP-related genes (i.e., the union of all the genes associated to all the KEs of the AOP). Enrichment *p*-values were adjusted using the false discovery rate (FDR) correction. AOP was considered significantly enriched with FDR-corrected *p*-value < 0.01.

KE Clustering and Construction of the Pulmonary Fibrosis Network: Similarities between the gene sets associated to each KE were evaluated by calculating the JI between all pairs of KEs (size of the intersection divided by the size of the union of the gene sets). The resulting similarity matrix was then transformed into a distance matrix and used to group the KEs using hierarchical clustering as implemented in the function *hclust* in R package stats, specifying the agglomeration method as "complete." The number of clusters was defined so that only KEs with the same gene sets associated to them (JI = 1) were assigned to the same group. The grouping obtained in this manner was used to perform the enrichment against KEs to avoid multiple testing against the same gene set as well as to enhance the network presentation of the PF AOP network. The unweighted PF AOP network was generated using gephi[107] by importing a graphml file generated with the function *graph_from_edgelist* from R package igraph.[108] KE groups from the clustering were added as attributes to the nodes and used to merge redundant nodes in gephi. Similarly, AOPs each KE is associated to were added as attributes and used to color the nodes.

Characterization of the AOP Fingerprints: *Transcriptomics data*: In vivo and in vitro transcriptomics data from MWCNT (Mitsui-7) exposures were selected from a previously published collection by Saarimäki et al.[67] The original data sets are available under GEO accession number GSE29042 (in vivo) and ArrayExpress entry EMTAB6396 (in vitro), while the

preprocessed data is available on Zenodo (https://doi.org/10.5281/zenodo.6425445). The in vivo data set comprised multiple doses and time points, while the in vitro data contained a single dose and time point exposure on four distinct cell lines representative different cell types of the lung. In each case, DEGs (filtered by an absolute fold change > 1.5 and Benjamini and Hochberg adjusted p-value < 0.05) obtained from Zenodo (https://doi.org/10.5281/zenodo.6425445) for each distinct comparison (i.e., combination of each dose and time point versus control in vivo and separate cell lines in vitro) were pooled together to obtain a distinct MOA of the exposure in vivo and in vitro, respectively.

AOP fingerprints: To produce the AOP fingerprint for the MWCNT exposures, enrichment analysis was performed using the Fisher's exact test as implemented in the function *enrich* from R package bc3net[106] separately against the AOP-associated gene lists and the KE-associated gene lists (KEs linked to the same set of genes were grouped to avoid multiple tests against the same set). An AOP was considered significantly enriched when the AOP itself and at least 33% (or minimum of 2 KEs when the length of the AOP was less than six) of its KEs were enriched with an FDR-corrected p-value < 0.05.

Selection and Testing of AOP-Driven Biomarkers: *Gene prioritization*: All human protein-protein interaction (PPI) edges were extracted from the UKS and used to create a robust gene–gene network. PPI/gene (product) interaction information could vary across data sources as well as the covered genes may differ. In addition, there was an innate bias in the data, where more data sources were available for "more investigated" genes and gene products. Because of this, it was decided not to apply a global threshold on how many sources need to support an edge,[109] but instead, a local threshold was applied. This ensured that also less investigated genes and gene products will be retained in the final robust gene–gene network, but their edges were less penalized by the number of supporting edges, than highly covered gene (product) nodes. For each node, the mean number of sources supporting all its connecting edges was estimated and only edges with at least the "mean number of sources" for a node were retained, which needed to be true for at least one of the nodes making up an edge. This was only done for GENE nodes, which were flagged as protein coding in Ensembl. The final robust human gene–gene network, consisted of 20 260 nodes, 806 250 edges, a network density of 0.0039. Due to the significant lower number of available sources for transcription factor—gene (product) data, all available sources were kept and scored equally. These edges were used to create a directed gene–gene network, consisting of 18 754 nodes, 363 649 edges and a network density of 0.001. On the so created gene–gene networks, for each node its degree, betweenness, eigenvector, and closeness centrality were estimated with NetworkX.[110] These measures were then used to rank the genes linked to the KEs in the context of individual KEs. The gene list for each KE was ranked based on each of the centrality measures (degree, betweenness, closeness, and eigenvector centrality) individually from most central to the least. The ranked lists were combined using the Borda method as implemented in the function *Borda* in R package TopKLists.[111]

Biomarker selection: The gene centrality-based ranking was then supplemented by a specificity ranking for the KEs of AOPs related to PF. A specificity score for the genes in the context of the KEs by dividing the occurrence of the gene in the KEs of PF AOPs by their occurrence in the KEs of other AOPs were calculated. A similar score was calculated at the level of AOPs (occurrence in PF AOPs/occurrence in other AOPs), as universal PF biomarkers were the target of the identification (i.e., prioritizing those that would be present in as many of the six PF AOPs as possible) while also being as specific as possible to PF. These ranks were again combined by the function *Borda* from R package TopKLists,[111] and a final round of the Borda method was applied to combine the lists of genes from each KE into one PF rank. The final rank was complemented with experimental evidence. It was assessed whether the genes were differentially expressed in the Mitsui-7 exposures in vivo and in vitro. It was also evaluated whether they were dose-dependently altered in the in vivo data as well as in an additional in vitro data set on Mitsui-7 exposure of a THP-1 macrophage model (originally published in Saarimäki et al.[58] and the preprocessed data available as GSE146708 in the previously published collection[67] available in https://doi.org/10.5281/zenodo.6425445). The dose-response modeling of the in vivo (GSE29042) and in vitro (GSE146708) datasets was performed by following the strategy implemented in the BMDx tool.[112] Particularly, for each gene present in the dataset, multiple models were fitted including linear, second order polynomial, hill, power, and exponential model. For each gene, the optimal model was selected as the one with the lowest Akaike Information Criterion (AIC). Genes with an optimal model with lack-of-fit p-values lower than 0.1 were removed from the analysis. The effective doses (BMD, BMDL, and BMDU) were estimated under the assumption of constant variance and by using a BMRF factor of 1.349 (corresponding to a minimum of 10% of difference with respect to the controls). Genes were further filtered based on the predicted doses. Genes with BMD or BMDU values extrapolated higher than the highest exposure dose were filtered. Moreover, genes whose ratio between the predicted doses was higher than the suggested values (BMD/BMDL> 20, BMDU/BMD> 20, and BMDU/BMDL> 40) were removed from the analysis. Genes passing the filters were considered to be dose-dependently altered. At this stage, the measurability and feasibility of the gene in the selected macrophage model was also considered. For instance, numerous collagen-encoding genes were ranked high, but would not be a meaningful target in a macrophage model. Moreover, a high coverage of PF KEs and the selection of genes with high specificity scores were emphasized. With these considerations, a subset of the genes with the following priority was selected: 1) genes that were deregulated both in vivo and in vitro, with most emphasis on dose-dependency; 2) genes that were deregulated in vitro, with most emphasis on dose-dependency; and 3) genes that were not significantly differentially expressed but were dose-dependent. Finally, after this initial selection driven by the rank and experimental evidence, additional candidate biomarkers that had a lower rank but were specific to KEs that would otherwise not have been covered by the selected candidates were included.

Cell culture: THP-1 cells (DSMZ no.: ACC 16) were grown in RPMI 1640 (Gibco, #21 875) + 10% inactivated FBS (Gibco, #10 270). Cells were cultivated in 75 cm^2 culture flasks at 37 °C with a humidified atmosphere of 5% CO_2. For all experiments, cells were seeded at a density of 1×10^5 cells mL^{-1} in 96 well plates and differentiated for 48 h with 25 nM PMA (phorbol-12-myristate-13-acetate, Sigma-Aldrich, #P1585). Cells were then left to rest for 24 h in fresh media containing no PMA prior to bleomycin exposures.

Cell viability assay: THP-1 cells were exposed to 0–10 µg mL^{-1} of bleomycin ready-made solution (Sigma-Aldrich, #B7216) and 0–100 mg mL^{-1} of Triclosan (Sigma-Aldrich, #72 779), for 6, 24 and 72 h. A WST-1 assay was then used to measure cell viability. Briefly, 10 µL of cell proliferation reagent WST-1 (Roche, #11 644 807 001) was added to each well. Cells were left to incubate with WST-1 for 3 h in a 37 °C, 5% CO2 incubator. Absorbance at 450 nm was then measured with a Spark microplate reader (Tecan). Results of the cell viability assay are available in File S2 and Figure S3, Supporting Information.

RT-qPCR: For each time point of 6, 24 and 72 h, THP-1 cells were exposed to 0, 2.5, 5, 10 and 100 µg mL^{-1} of bleomycin ready-made solution (Sigma-Aldrich, #B7216). Media was removed and cells were washed briefly with 50 µL of PBS. 100 µL of lysis buffer from the QIAGEN RNeasy mini kit (Qiagen, #74 104) was added to each well to lyse the cells. Three wells (300 µL) were pooled to create one sample, and there were five samples for each concentration at each time point. Total RNA was then extracted from these samples using the QIAGEN RNeasy mini kit (Qiagen, #74 104). DNase treatment was performed using TURBO DNA-free Kit (ThermoFisher, #AM1907) according to the manufacturer's protocol. cDNA was synthesized from 100 ng of RNA, using the high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, #4 368 813), according to manufacturer's instructions. Expression levels of target genes were determined by qRT-PCR using CFX96 Touch Real-Time PCR Detection System (BioRad) with 10 µL of iQ Multiplex Powermix (Bio-Rad, #1 725 849), 5 µL of cDNA diluted fivefold, 2.5 µL of nuclease-free (NF) water (not DEPC-Treated, ThermoFisher, #AM9930) in a 20 µL reaction, together with 2.5 µL of single (1 µL assay + 1.5 µL NF water) or multiplexed (0.5 µL of each assay) PrimePCR Probe Assays (Bio-Rad) as followed with single or multiplex reactions grouped in parentheses and formatted as *Gene*/UniqueAssayID:

(*ACTB*/qHsaCEP0036280), (*SMAD7*/qHsaCEP0050142, *MMP9*/qHsaCIP0028098, *GDF15*/qHsaCEP0051579, *CTSK*/qHsaCIP0030907, *PLOD2*/qHsaCEP0052848), (*CXCL2*/qHsaCEP0058163, *LTBP4*/qHsaCEP00249-31, *TGFB3*/qHsaCEP0058244, *RCN3*/qHsaCEP0057804, *MMP7*/qHsaCEP0052037), (*SPP1*/qHsaCEP0058179, *FN1*/qHsaCEP0050873, *LTBP3*/qHsaCEP0053782, *RSAD2*/qHsaCIP0031596, *CCL7*/qHsaCEP0058033), (*IL8*/qHsaCEP0053894, *MMP19*/qHsaCEP0051244, *TWIST1*/qHsaCEP-0051221, *PLK3*/qHsaCIP0027687, *CXCL10*/qHsaCEP0053880), (*LOX*/qHsaCEP0050731, *PTX3*/qHsaCEP0033071, *TGFBI*/qHsaCEP0058394, *CCL2*/qHsaCIP0028103, *TGFB1*/qHsaCIP0030973).

Fold change (FC) values from RT-qPCR data were calculated using the comparative CT($2^{-(ddCt)}$) method.[113] The FC values were log2 transformed ($\log_2$(FC)). For each gene and for each concentration, an outlier detection was performed by removing all the samples with $\log_2$(FC) values above or below the 75th and 25th percentiles of the distribution. C_t values, dC_t values, FC values and $\log_2$(FC) values are available in File S2, Supporting Information, along with ANOVA tables and tukey HSD posthoc test results. The $\log_2$FC expression of the genes as compared to the untreated controls are plotted in Figure S4, Supporting Information.

Dose-dependent modeling: A dose-response analysis of the $\log_2$(FC) values derived from the PCR experiments was performed. For each gene, multiple models were fitted, including linear, hill, power, polynomial, exponential, log-logistic, Weibull, and Michaelis–Mentel models. The optimal model was selected as the one with the lowest AIC. The BMD estimation was performed under the assumption of constant variance. The BMR was identified by means of the standard deviation approach with a BMRF of 1.349. Only genes with lack-of-fit *p*-value >0.10 and with estimated BMD, BMDL and BMDU values were considered relevant.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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